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Filing Date	Feb. 22, 2002
First Named Inventor	Michael Brandt
Art Unit	1646
Examiner Name	Gyan Chandra
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ENCLOSURES *(Check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
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Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Robert P. Hoag
Signature	Robert P. Hoag
Date	May 3, 2006

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Typed or printed name	Robert P. Hoag		
Signature	Robert P. Hoag	Date	May 3, 2006

In re patent application

Inventors: Brandt et al

Application No.: 10/081,309

Filed: February 22, 2002

For: **PEG CONJUGATES OF NK4**

Hoffmann-La Roche Inc.
340 Kingsland Street
Nutley, NJ 07110
May 3, 2006

REQUEST TO CHARGE DEPOSIT ACCOUNT – BRIEF ON APPEAL

Mail Stop: Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith is a Brief on Appeal in the above-identified application.

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X The Commissioner is hereby authorized to charge any additional fees associated with this communication or credit any overpayment to Deposit Account No. 08-2525.

This sheet is provided in duplicate.

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143911

DEPOSIT ACCOUNT
NO. 08-2525
OUR ORDER NO. 6763

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application

Inventor(s): Brandt, et al.

Application No.: 10/081,309

Filed: February 22, 2002

Technology Center: 1644

Attorney Docket No. 20859 US

Art Unit: 1646

Examiner: Gyan Chandra

FOR: **PEG CONJUGATES OF NK4**

APPEAL BRIEF

Nutley, New Jersey 07110
May 3, 2006

Mail Stop: Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Further to the Notice of Appeal which was filed on March 6, 2006, Applicants submit this Appeal Brief appealing the final Office Action dated September 2, 2005, and Advisory Action dated March 24, 2006, rejecting claims 1-2, 4-6, 8 and 12-15. Submitted herewith (in duplicate) is the appropriate document authorizing payment of the required fee in connection with the filing of this Appeal Brief under 37 C.F.R. § 41.20(b)(2).

I. REAL PARTY IN INTEREST

The rights to this application have been assigned to Hoffmann-La Roche Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 3, 7 and 9-11 were withdrawn by the Examiner as nonelected. Claims 1-2, 4-6, 8 and 12-15 were rejected by the Examiner under 35 U.S.C. Sect. 103(a). A copy of appealed claims 1-2, 4-6, 8 and 12-15 is attached hereto in the Claims Appendix.

The appealed grounds for rejection apply to all of the rejected claims.

IV. STATUS OF AMENDMENTS

No amendments were filed after the final Office Action of September 2, 2005 which rejected claims 1-2, 4-6, 8 and 12-15.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The claimed subject matter is drawn to a conjugate consisting of a NK4 molecule and a polyethylene glycol group having a molecular weight of about 20-40 kDa. The claimed subject matter is also drawn to said conjugate wherein the polyethylene glycol group:

i) has the formula $-CO-(CH_2)_X-(OCH_2CH_2)_mOR$, wherein X is 2 or 3; m is from about 450 to about 950; and R is $(C_1\text{-}C_6)$alkyl;

ii) is monomethoxy polyethylene glycol; and

iii) forms an amide group with one of the amino groups of the N-terminal fragment of the NK4 fragment.

Support for the claimed subject matter can be found throughout the specification, particularly at page 3, paragraph [0008] to page 25, paragraph [0086].

VI. GROUNDS OF REJECTION

Claims 1-2, 4-6, 8 and 12-13 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Namiki et al (EP 0816381 A1, published on 07/01/1998) in view of Date et al (Oncogene, 17:3045-3054, 1998) and Gaertner et al (Bioconjugate 7:38-42, 1996). According to the Examiner, it would have been obvious to one skilled in the art, in light of the above references, "to experimentally determine which types of pegylation would result in a protein with improved characteristics (the claimed conjugate(s))".

Claims 14 and 15 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Namiki et al (EP 0816381 A1, published on 07/01/1998) in view of Date et al (Oncogene, 17:3045-3054, 1998) and Gaertner et al (Bioconjugate 7:38-42, 1996) and further in view of Veronese (US patent 6,528,485 B1).

A copy of the final Office Action dated September 2, 2005 and the Advisory Action of March 26, 2006, as well as copies of the references cited by the Examiner therein, are attached hereto in the Evidence Appendix.

VII. ARGUMENT

1. Obviousness Rejection Under 35 U.S.C. § 103(a)

A. Claims 1-2, 4-6, 8 and 12-13

(1) The Examiner's Rejection

In the pending rejection, Namiki et al is cited for teaching how to modify hepatocyte growth factor (HGF) by attaching a polyethylene glycol (PEG), and a pharmaceutical composition comprising the PEG modified HGF (page 12, line 37-40). The Examiner admits that Namiki does not teach NK4 nor the attachment of PEG with a molecular weight of 20 to 40 kDa, nor monomethoxy PEG. The Date reference is cited for allegedly disclosing that HGF comprise, in part, a 4 kringle fragment as having NK-4. Gaertner is cited as disclosing attaching PEG at the amino terminus of proteins and, therefore, allegedly suggesting a 5 to 40 kDa PEG should be attached to a protein (for an improved bioavailability), at a single attachment point using an oxime bond. According to the Examiner, it would be obvious to combine Namiki with Date and Gaertner to attach PEG molecules to the N-terminus amino acid of NK4.

(2) Summary of Applicants' Arguments In Response

Claims 1-2, 4-6, 8 and 12-13 are not obvious because: (1) there is no structural similarity between the pegylated compounds disclosed in Namiki and Gaertner and the claimed invention for the reasons stated below; (2) there is a lack of motivation to combine Gaertner with Namiki and Date for the reasons stated below; and (3) even if there was motivation to combine Gaertner with Namiki and Date, the Gaertner reference a) does not teach the NK4 molecule of the present invention and teaches away from consistent increased bioavailability via large PEG attachment (Claim 1) and b) actually teaches away from forming an amide bond via PEGylation , and c)

additionally teaches away from a single point attachment of a large PEG molecule at said amide bond, instead teaching that it should be attached at an oxime bond – which is contrary to the amide bond formation of Applicant's invention (Claim 2).

(3) The Law of Obviousness Under 35 U.S.C. § 103

"A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the **differences** between the subject matter sought to be patented and the prior art are such that the subject matter **as a whole** would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains" (emphasis added). 35 U.S.C. § 103(a).

Under 35 U.S.C. § 103, "the scope and content of the prior art are to be determined; **differences between the prior art and the claims at issue are to be ascertained**; and the level of ordinary skill in the pertinent art resolved." *Graham v. John Deere*, 383 U.S. 1, 148 U.S.P.Q. 459, 467 (1966) (emphasis added). The following tenets of patent law must be considered and adhered to when applying 35 U.S.C. § 103:

> (A) The claimed invention must be considered **as a whole**;
> (B) The references must be considered **as a whole** and **must suggest the desirability** and thus the obviousness of making the combination; and
> (C) The references must be viewed **without the benefit of impermissible hindsight** vision afforded by the claimed invention.
> *Hodosh v. Block Drug Co., Inc.*, 786 F.2d 1136, 1143 n.5 (Fed. Cir. 1986).

For claims directed to chemical compounds, "obviousness requires 'structural similarity between claimed and prior art subject matter . . . **[but only] where the prior art gives reason or motivation to make the claimed compositions**.'" *Yamanouchi*

Pharmaceutical Co., Ltd. et al. v. Danbury Pharmacal, Inc. et al., 231 F.3d 1339, 1343 (Fed. Cir. 2000) (emphasis added); *In re Mills*, 916 F.2d 680, 682 (Fed. Cir. 1990) (the mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination); *Al-Site Corp. v. VSI Int'l Inc.*, 174 F3d 1308, 1323-24 (Fed. Cir. 1999) (obviousness requires some motivation or suggestion to modify or combine the prior art teachings but the fact that a claimed invention was within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish obviousness).

In addition, "[t]he fact that **a claimed compound may be encompassed by a disclosed generic formula does not by itself render that compound obvious**." *In re Baird* 16 F.3d 380, 382 (Fed. Cir. 1994) ("While the Knapp formula unquestionably encompasses bisphenol A when specific variables are chosen, there is nothing in the disclosure of Knapp suggesting that one should select such variables.") (emphasis added). "[H]omology should not be automatically equated with prima facie obviousness." *In re Langer*, 59 C.C.P.A. 1256, 1260, 465 F.2d 896, 899 (CCPA 1972) (Claims to a polymerization process using a sterically hindered amine were held unobvious over a similar prior art process because the prior art disclosed a large number of unhindered amines and only one sterically hindered amine (**which differed from a claimed amine by 3 carbon atoms**), and therefore the reference **as a whole** did not apprise the ordinary artisan of the significance of hindered amines **as a class**). *See also In re Jones*, 958 F.2d 347, 350 (Fed. Cir. 1992) (reversing obviousness rejection of novel dicamba salt with acyclic structure over broad prior art genus encompassing claimed salt, where disclosed examples of genus were dissimilar in structure, lacking an ether linkage or being cyclic); and *Ex parte Burtner and Brown*, 121 U.S.P.Q. 345, 347 (Bd. of App. 1951) (holding claimed alcohols patentable over prior art compounds differing by a –CH2- group). Copies of the above-referenced cases are attached hereto in the Case Appendix. In the *In re Langer* case, the court held that:

> We view appellants' invention "**as a whole**" as being the use of sterically hindered amines as opposed to unhindered amines in a known process to solve a particular problem . . . . We do not think the **[prior art]** patent provides a basis for the use of sterically hindered amines **as a class**, or of any of the amines encompassed by appellants' claims . . . The presence in the reference of an **isolated** hindered amine . . . does not, by itself, apprise the ordinary artisan of the significance of hindered amines **as a class** . . . . **when "all of the disclosures in a reference" are considered**, the overall suggestion to emerge from the prior art reference may be contrary to that which might appear from **an isolated portion of the reference**. *In re Langer*, 465 F.2d at 899 (emphasis added).

The test for obviousness is not whether the features of a secondary reference may be bodily incorporated into the structure of the primary reference... Rather, the test is what the combined teachings of those references would have suggested to those of ordinary skill in the art." *In re Keller, 642 F.2d 413, 425, 208 USPQ 871,881 (CCPA 1981).* The clamed combination cannot change the principle of operation of the primary reference or render the reference inoperable for its intended purpose, MPEP § 2141.02 (prior art must be considered in its entirety, including disclosures that teach away from the claims).

It is improper to combine references where the references teach away from their combination. *In re Grasselli,* 713 F.2d 731,743, 218 USPQ 769, 779 (Fed. Cir. 183) (The claimed catalyst which contained both iron and an alkali metal was not suggested by the combination of a reference which taught the interchangeability of antimony and alkali metal with the same beneficial result, combined with a reference expressly excluding antimony form, and adding iron to, a catalyst).

The prior art must suggest the desirability of the claimed invention "There are three possible sources for a motivation to combine references: the nature of the problem to be solved, the teachings of the prior art, and the knowledge of persons or ordinary skill in the art. "*In re Fouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998) (The combination of the references taught every element of the claimed invention, however without a motivation to combine, a rejection based on a

prima facie case of obvious was held improper). The level of skill in the art cannot be relied upon to provide the suggestion to combine references. *Al-Site Corp. v. VSI Int'l Inc.*, 174 F.3d 1808, 50 USPQ2d 1161 (Fed. Cir. 1999).

The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990) (claims were directed to an apparatus for producing an aerated cementitious composition by drawing air into the cementitious composition by driving the output pump at a capacity greater than the feed rate. The prior art reference taught that the feed means can be run at a variable speed, however the court found that this does not require that the output pump be run at the claimed speed so that air is drawn into the mixing chamber and is entrained in the ingredients during operation. Although a prior art device "may be capable of being modified to run the way the apparatus is claimed, there must be a suggestion or motivation in the reference to do so. " 916 F.2d at 682, 16 USPQ2d at 1432.). See also *In re Fritch*, 972 F.2d 1260, 23 USPQ2d 1780 (Fed. Cir. 1992) (flexible landscape edging device which is conformable to a ground surface of varying slope not suggested by combination of prior art references).

Finally, the fact that the claimed invention is or might be within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish *prima facie* obviousness. A statement that modifications of the prior art to meet the claimed invention would have been "well within the ordinary skill of the art at the time the claimed invention was made" because the references relied upon teach that all aspects of the claimed invention were individually known in the art is not sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teachings of the references. *Ex parte Levengood*, 28 USPQ2d 1300 (Bd. Pat. App. & Inter. 1993). See also *In re Kotzab*, 217 F.3d 1365, 1371, 55 USPQ2d 1313, 1318 (Fed. Cir. 2000) (Court reversed obviousness rejection involving technologically simple concept because there was no finding as to the principle or specific understanding with

the knowledge of a skilled artisan that would have motivated the skilled artisan to make the claimed invention); *Al-Site Corp. v. VSI Int'l Inc.*, 174 F.3d 1808, 50 USPQ2 1161 (Fed. Cir. 1999) (The level of skill in the art cannot be relied upon to provide the suggestion to combine references).

(4) The Pending Claims Are Not Obvious

In the Office Actions of March 30, 2005, September 2, 2005 and the Advisory Action of March 24, 2006, the Examiner's analysis of rejected claims 1-2, 4-6, 8 and 12-13 focuses on the terms, limitations and elements of claim 2. Applicant respectively asserts that all of the rejected pending claims are not obvious and thus are patentable. Claim 1 and its dependent claims are patentable because, as set forth infra, the cited references (Namiki, Date, and Gaertner) do not anticipate nor teach or suggest, singly or in combination, the attachment of a 20 to 40 kDa polyethylene glycol group to a NK4 molecule to form the claimed conjugate. Claim 2 is patentable because, as set forth infra, the cited references do not anticipate nor teach, singly or in combination, the conjugate of claim 1 wherein the polyethylene glycol group:

iv) has the formula – CO – $(CH_2)_X$ – $(OCH_2CH_2)_mOR$, wherein X is 2 or 3; m is from about 450 to about 950; and R is $(C_1$-$C_6)$alkyl;

v) is monomethoxy polyethylene glycol; and

vi) forms an amide group with one of the amino groups of the N-terminal fragment of the NK4 fragment.

In the present application, the Examiner asserts that Namiki et al teach how to modify hepatocyte growth factor (HGF) by attaching a polyethylene glycol (PEG), as well as a pharmaceutical composition comprising the PEG modified HGF (page 12, line 37-40). However, Applicants note that Namiki only teaches the modification of HGF by attaching monoethoxy linear and branched PEG(s) at the N-terminus amino acids to improve the clearance and in vivo pharmacokinetics of HGF (page 2, line 49-57), in direct contrast to Applicant's claim 5 (monomethoxy PEG). The Examiner admits that

Namiki does not teach NK4 nor the attachment of PEG with a molecular weight of 20 to 40 kDa. Accordingly, Namiki does not anticipate nor teach Applicant's claim 1 and its dependents nor claim 2.

In the pending rejection, the Date reference is relied upon for allegedly disclosing that HGF comprises, in part, a 4 kringle fragment as having NK4. At best therefore, the combined references of Namiki and Date would not teach attachment of PEG with a molecule weight of 20 to 40 kDa on the NK4 molecule (as in Applicant's claim 1 and its dependents), much less attachment of such size PEG at the N-terminus location of NK4 (claim 2).

The Gaertner reference is relied upon for allegedly disclosing attaching PEG at amino terminus of certain proteins (IL-8 and G-CSF). Gaertner is therefore used by the Examiner in combination with Date and Namiki above, to support a rejection based on a suggestion that a PEG in size from 5 to 40 kDa should be attached to a protein (for an improved bioavailability) at a single attachment point using an oxime bond. While this rejection would, presuming *arguendo* proper motivation to combine as well as appropriately applied and factually based, arguably cover the elements of claim 1 and some of its dependents, this rejection does not cover all the elements of claim 2, which requires an amide bond. As such, Applicants submit that Claim 2 is not even *prima facie* rejected and thus should be allowed.

In attempting to show obviousness, the Examiner impermissibly picks and chooses only selective elements of the above cited references, while ignoring other elements, to arrive at the instant claimed invention. Such application of hindsight is impermissible. For example, the Examiner selects the large PEG attachment teaching of Gaertner, but ignores that the reference teaches attaching the PEG to a specific serine or threonine site via an oxime bond. Indeed, the Examiner gives no weight to Gaertner's requirement that its method relies on "the ability to generate a reactive carbonyl group in place of the terminal amino group... to form a stable oxime bond."

(Abstract). This statement directly affects the applicability of Gaertner to Applicant's Claim 2. The Examiner also ignores Gaertner's disclosure that a large PEGylation (20 kDa) of G-CSF resulted in less biological activity. This disclosure affects the motivation to combine Gaertner with Namiki with regard to Applicant's invention as claimed in Claim 1 and its dependents.

The Gaertner (1996) reference discloses pegylation of certain proteins only at N-terminal serine or threonine sites (see e.g., Il-8 usage in Gaertner because "it has the appropriate N-terminal residue"). If the serine or threonine site is not readily available to pegylate via an oxime bond, Gaertner teaches that additional procedural steps are required to result in a serine or threonine site to pegylate. For example, Gaertner requires enzymatic cleavage/digestion of Met-Thr-Pro for the site specific pegylation of G-CSF to expose the threonine. Gaertner also requires selective modification of the alpha amino group by metal-catalyzed transamination of Met-Arg-Pro for the site specific pegylation of IL-1ra. Gaertner acknowledges that these additional steps have drawbacks of exposing the protein to a metal, side product formation and reactions and lower yields. Gaertner also acknowledges that not all large PEGylations increase biological activity.

In other words, Gaertner et al. merely discloses a method for the PEGylation of a terminal serine and threonine amino acid via oxime bond formation. If no serine or threonine residue is available at the N-terminus of the protein, Gaertner requires one of two procedures, 1) either additional steps are required to uncover a serine residue present within the last two amino acids of the protein (example with G-CSF) ii) or 2) additional steps involving harsh oxidation conditions have to be employed (example with IL-1ra) yielding a plurality of side products and reducing the overall yield.

The PEGylation according to Gaertner et al. is thus accomplished by the formation of an oxime bond between an N-terminally introduced carbonyl group in the IL-8 and G-CSF protein and an aminooxy functionalized PEG. Gaertner does not teach

PEGylation of NK4 or even HGF (as in claim 1) and its requirement for an oxime bond is in direct contrast to the formation of an amide bond as reported in claim 2 of the current invention. Gaertner et al. also only teaches the attachment of linear PEG with a molecular weight of approx. 20 kDa and thus does not teach attachment of linear or branched PEG (as is claimed in Applicant's claim 7) nor of a 20 kDa to 40 kDa PEG attachment to NK4 (as is claimed in Applicant's claim 1). Gaertner does not teach, nor disclose, amide bond formation or pegylation of the N-terminal lysine group (as is specifically claimed in Applicant's claim 2 and claim 8). Indeed Gaertner teaches away from lysine as a pegylation site by requiring the pegylation site to be serine or threonine, even at the cost of side product formation and reduced yield, and thus teaches away, at the very least, from claims 2, 8 and 13 of Applicant's invention.

As Gaertner admits, the PEGylation presents a big problem if the attachment sites are not precisely controlled, as this affects protein stability and functionality. In other words, per Gaertner, it is serine or threonine, or nothing for pegylation. As such, it is hard to imagine combining Gaertner's teaching with Namiki in any way other than for site specific pegylation at specific serine or threonine sites – or in other words, to form oxime bonds. Claim 2 of Applicant's invention specifically claims forming an amide bond for the pegylation. Gaertner specifically notes with regard to amide bond formation for the attachment of a PEG: "while with other conventional methods, the extent of modification of the different amino groups, which is less reproducible, has to be taken into account".

Furthermore, it is hard to imagine combining Gaertner with Namiki at all, as each reference is concerned with different size pegylation of different proteins at different locations. Gaertner does not teach, disclose or suggest PEGylation of NK4 or even HGF, much less what size of PEGylatyion to attach to the NK4 protein, but merely teaches PEG attachment to IL-8 and G-CSF. Namiki, as admitted by the Examiner, does not teach NK4 nor the attachment of PEG with a molecular weight of 20 to 40 kDa. Accordingly, Namiki does not anticipate nor teach Applicant's claim 1 and its

dependents nor claim 2. Since Gaertner does not teach PEG attachment of any size to NK4, the combination of Gaertner and Namiki does not teach 20 to 40 kDa PEG attachment to NK4. Thus, even if one was motivated to combine the two references, the combination of Gaertner with Namiki (and Date) would not teach, disclose or suggest the conjugate of Applicant's claim 1 or its dependents thereto.

Indeed, Gaertner contrasts the prior art attachment of small pegylated group (5kD) to one or more amino groups to get increased in vivo bioavailability with its own method to attach a "sufficient(ly) sized PEG" (20kd) to oxidized IL-8 polymers via oxime bond. Thus, far from the Examiner's assertion that there would be a motivation to combine Gaertner with Namiki, there is in fact not only a lack of such motivation, but indeed a teaching away from the prior art methods of small PEG attachments at amide bonds (Namiki) to a single sufficiently large PEG attachment at an oxime bond (Gaertner). Simply stated, the two references are at odds with each other and cannot be combined, especially with regard to Applicant's claim 2, which requires the PEG attachment to the NK4 molecule at an amide bond.

Without Gaertner, the combination of references adduced by the Examiner do not cover all elements claimed by Applicant. The Examiner has admitted and acknowledged such on the record. Namiki and Date do not teach, disclose or suggest Applicant's claim 1, much less any of its dependents. Claim 2 is likewise not taught, disclosed or suggested by Namiki and Date. When each reference is viewed in its entirety and as a whole (See, e.g., Hodosh), the references in combination do not suggest the obviousness of making the combination (conjugate) of claim 1, its dependents and claim 2.

The other cited references of record support the lack of motivation to combine Namiki and Gaertner. Indeed, as disclosed by Applicants' specification, the Mehvar (2000) reference (which was published four years after Gaertner) states that conjugation of different polyethylene glycols to IL-8 and G-CSF as well as other

interleukins results in the production of molecules with impaired properties (specification, paragraph 5). Furthermore, Francis et al. (1998) posits that "PEGylation of proteins is always based on trial and error and virtually all parameters of such a PEGylation can have a surprising and very profound effect on the functionality of the product" (specification, paragraph 6). Finally, as disclosed in Applicant's specification, Reddy (2000) states that each protein requires different optimization chemistry and therefore the influence of PEGylation cannot be predicted. In other words, the art of record clearly indicates that this is a "trial and error" field. Each protein is different and requires different chemistry for PEGylation. The applicability of Gaertner's disclosure of the PEGylation of IL-8 and G-CSF to the PEGylation of a different protein, such as NK4, cannot be predicted: "PEGylation of proteins is always based on trial and error." In light of such teachings, one cannot pick and choose a claim element from Gaertner to add to a claim element of Namiki to arrive at Applicant's claimed invention, whether it is the conjugate of claim 1 (and its dependents) or the conjugate of claim 2. Instead, the entire teaching of the references must be considered.

Like the cases discussed above, there is no suggestion or motivation to select a compound close in structure to the claimed invention, indeed there is no statement or teaching alleged by the Examiner that the proteins PEGylated in Gaertner are similar (and to what degree) to the NK4 protein of Applicant's invention, and then to modify same by 20 to 40 kDa PEG attachment (Claim 1 of Applicant's invention). Moreover, there is no suggestion or motivation to further modify such a protein (NK4) by such a pegylation via an amide bond to arrive at the claimed invention (Claim 2 of Applicant's invention). As in the *In re Langer* case, both Namiki and Gaertner fail to "apprise the ordinary artisan of the significance of the [Applicants' compounds] as a class." *In re Langer*, 465 F.2d at 899. In addition, there is no structural similarity between the bonds disclosed in Namiki (amide) and Gaertner (oxime) which decreases motivation to continue and the applicability of these references together as to Applicants invention, especially with regard to Claim 2.

(a) The Reference And The Claimed Invention Must Be Viewed As A Whole

The Examiner asserts that the differences enumerated above between the claimed invention and Gaertner and Namiki is irrelevant based on alleged high skill in the art of pegylation of proteins at N-terminus. *See* the final Office Action and Advisory Action. The Examiner is not acknowledging the entire teaching/procedure of Gaertner, in particular, its specific requirement that there has to be a "reactive carbonyl group in place of the terminal amino a group ... to form a stable oxime bond in the PEGylated group." The Examiner is also not acknowledging that Gaertner does not teach nor disclose PEGylation of NK4, but merely such proteins as IL-8 and G-CSF. The Examiner also glosses over Gaertner's disclosure that a 20kDa mPEGylation of G-CSF to the N-terminus resulted in a 2-3 fold **decrease** of biological activity. The Examiner is thus not viewing Gaertner as a whole.

However, the Courts have ruled that prior art references must be considered **as a whole** and **must suggest the desirability** and thus the obviousness of making the combination. *Hodosh v. Block Drug Co., Inc.*, 786 F.2d 1136, 1143 n.5 (Fed. Cir. 1986). In addition, the prior art references must be viewed **without the benefit of impermissible hindsight** vision afforded by the claimed invention. *Id.*

In considering motivation in the obviousness analysis, the problem examined is not the specific problem solved by the invention but the general problem that confronted the inventor before the invention was made. See, e.g., Cross Med. Prods., Inc. v. Medtronic Sofamor Danek, Inc., 424 F.3d 1293, 1323 (Fed. Cir. 2005) ("One of ordinary skill in the art need not see the identical problem addressed in a prior art reference to be motivated to apply its teachings."); Ecolochem, Inc. v. S. Cal. Edison Co., 227 F.3d 1361, 1372 (Fed. Cir. 2000) ("Although the suggestion to combine references may flow from the nature of the problem, **'[d]efining the problem in terms of its solution reveals improper hindsight in the selection of the prior art relevant to obviousness**.'" (internal citation omitted) (quoting Monarch Knitting Mach. Corp. v.

Sulzer Morat GmbH, 139 F.3d 877, 881 (Fed. Cir. 1998))); In re Beattie, 974 F.2d 1309, 1312 (Fed. Cir. 1992) ("[T]he law does not require that the references be combined for the reasons contemplated by the inventor."); Princeton Biochemicals, Inc. v. Beckman Coulter, Inc., 411 F.3d 1332, 1337 (Fed. Cir. 2005) (characterizing the relevant inquiry as "[would] an artisan of ordinary skill in the art at the time of the invention, confronted by the same problems as the inventor and with no knowledge of the claimed invention,[] have selected the various elements from the prior art and combined them in the manner claimed"); see also Graham, 383 U.S. at 35 (characterizing the problem as involving mechanical closures rather than in terms more specific to the patent in the context of determining the pertinent prior art). Therefore, the "motivation-suggestion-teaching" test asks not merely what the references disclose, but whether a person of ordinary skill in the art, **possessed with the understandings and knowledge reflected in the prior art, and motivated by the general problem facing the inventor**, would have been led to make the combination recited in the claims. See Cross Med. Prods., 424 F.3d at 1321-24. From this it may be determined whether the overall disclosures, teachings, and suggestions of the prior art, and the level of skill in the art—i.e., the understandings and knowledge of persons having ordinary skill in the art at the time of the invention—support the legal conclusion of obviousness. See Princeton Biochemicals, 411 F.3d at 1338 (pointing to evidence supplying detailed analysis of the prior art and the reasons one of ordinary skill would have possessed the knowledge and motivation to combine).

The problem facing one of ordinary skill in the art at the time of the Applicant's claimed invention was how to attach a large PEG group (20kDa to 40kDa) to the NK4 protein to hopefully improve bioavailability etc. of the protein via the resulting conjugate. More specifically, the problem concerned how to attach a large mono-PEG group at a specific site on the NK4 protein in order to hopefully improve bioavailability etc of the protein/conjugate. At this point in time, the art taught that PEGylation of cytokines and other proteins typically resulted in substantial reduction of bioactivity (See e.g. Francis et al., 1998 and Specification, paragraph (0006)) and that the PEGylation process was "always based on trial and error and virtually all parameters of

such a PEGylation can have a surprising and very profound effect on the functionality of the product." (Id.) In other words, each protein is different and requires therefore different optimization chemistry and that therefore, the influence and results of PEGylation cannot be predicted. (See e.g. Reddy et al., 2000). Even the size of the PEG group to be attached to the targeted protein was unpredictable: while Gaertner reports some success with 5 to 40 kDa PEG attachments to IL-8, G-CSF and IL-1ra, Gaertner additionally discloses that a 20kDa mPEGylation of G-CSF to the N-terminus resulted in a 2-3 fold **decrease** of biological activity. (See page 43).

Here, the Examiner has chosen in hindsight Gaertner's teaching of attaching 20Kda PEGylation via oxime bonds of the proteins IL-8 and G-CSF to teach PEGylation of NK4 protein via amide bond formation when used in combination with Namiki and Date. However, one of ordinary skill in the art, at the time of applicant's invention, would not have combined Gaertner with Namiki because 1) Gaertner does not teach PEGylation of NK4 or HGF (Claim 1), and 2) Gaertner does not teach amide bond formation to PEGylate (Claim 2). On the contrary, Gaertner teaches 1) only specific proteins for PEGylation (IL-8, G-CSF and IL-1ra), 2) only oxime bond formation for said PEGylation and furthermore 3) specifically points out problems with site-specific pegylation at sites other than serine and threonine. Indeed, Gaertner even discloses that a 20kDa mPEGylation of G-CSF to the N-terminus resulted in a 2-3 fold **decrease** of activity (which would surely teach away from the combination of a 20kDa to 40kDa PEGylation of NK4 as in Applicant's Claim 1). Other references available to one of ordinary skill in the art likewise indicate the complexity of PEGylating different proteins at different sites. Moreover, the Examiner is presuming that what works for G-CSF and IL-8 in Gaertner will somehow obviously also work for NK4, a presumption which is not alleged to even be known by one of ordinary skill in the art. This presumption is contradicted by the references of record (Francis et al., Reddy et al., and even Gaertner) that state that the influence of pegylation on any one protein cannot be predicted. For example, the use of IL-8 as a model protein by Gaertner is potentially misleading, as it was used because "it happens to have the appropriate N-terminal residue" ie serine or threonine to form an oxime bond. (See pg 44). However, even the

PEGylation of IL-8 is not predictable. Mehvar et al. (2000) reported, four years after Gaertner, that conjugation of different polyethylene glycol groups (PEGs) to IL-8 and G-CSF (the proteins of Gaertner) results in the production of molecules with impaired properties. (Specification, paragraph (0005). As such the teachings of Gaertner and the art at the time of Applicant's invention would not lead one of ordinary skill in the art to the invention of Applicant's claim 1, much less its dependents or claim 2.

In addition to the references cited above, the references cited by Applicant and the Examiner in the Office Actions also show the unpredictability of PEGylating even one protein via different methods and PEG sizes, thus demonstrating the "trial and error" of the field:

1996: Gaertner - Teaches PEGylation of 5kDa to 40kDa via oxime bond of G-CSF and IL-8 proteins. "This method relies on the ability to generate a reactive carbonyl group in place of the terminal amino group ... to form a stable oxime bond." Not all such sized PEGylations increase biological activity: a 20 kDa mPEGylation of G-CSF "to the N-terminus resulted in a 2-3 fold **decrease** of the activity."

1998: Namiki - Teaches modification of HGF by monoethoxy PEGylation to N-terminus. Does not teach NK4 nor the attachment of PEG with a molecular weight of 20 to 40 kDa, nor monomethoxy PEG.

1998: Date - HGF asserted to comprise in part 4 kringle fragment containing NK4.

2000: Reddy - Influence of PEGylation cannot be predicted. Each protein requires different optimization chemistry.

2000: Mehvar - Conjugation of "different PEG" groups to IL-8 and G-CSF results in production of molecules with "impaired properties".

2001: Applicant's invention priority date (Feb 23, 2001).

The Examiner attempts to bypass this hodgepodge of teachings by stating that "it is also known that the same protein may be suboptimally active when PEGylation is achieved at N-terminus and that therefore site-specific PEGylation is well practiced." This statement begs the point of the above references, which a) first do not all teach the same site-specific attachment for PEGylation, b) do not all teach the same PEG group, c) do not all teach the formation of the same bond for PEG attachment (oxime vs amide) , and perhaps most importantly d) do not even teach PEG attachment to the same proteins! The Gaertner reference teaches N-terminus PEGylation at serine or threonine of G-CSF and IL-8 proteins via oxime bond and teaches away from amide bond formation (must remove amino acids to get to serine/threonine and form oxime bond). The attachment of a large PEG group of 20 to 40 kDa to NK4 is not anticipated and the art teaches, as a whole and in combination, that the PEGylation of protein filed is one of "trial and error" and is unpredictable.

The combination of Mehvar and Gaertner for example illustrates this point. The 1996 Gaertner reference teaches 5 to 40 kDa PEGylation of IL-8 and G-CSF and purportedly improved biological activity. Contrast this teaching with Mehvar, which teaches that the conjugation of "different PEG" groups to IL-8 and G-CSF results in production of molecules with "impaired properties". If just changing the PEG to be attached results in such a different result in the biological activity of the exact same protein, what is the predictability for a conjugate wherein not only is the PEG to be attached changed, but also the protein to which it is attached? Yet this is what the Examiner is attempting to do by combining the teachings of Gaertner with Namiki (and Date) to render obvious Applicant's Claim 1, its dependents and Claim 2. One of ordinary skill in the art would have appreciated and understood the unpredictability of combining two such disparate references in such a way that would involve a different PEG size than that of Namiki (from Gaertner) and a different protein than that of Gaertner (Namiki in view of Date) to attach said different sized PEG to form a new conjugate (Applicant's Claim 1). If Mehvar and Gaertner cannot even be combined predicatably when both involve the pegylation of the exact same proteins, then surely

the combination of Gaertner and Namiki cannot be predictably combined. As such, Claim 1 and its dependents thereto are non-obvious and patentable.

The above analysis and conclusion of non-obviousness is even more apparent with regard to Claim 2. Far from being something well known and obvious to one of ordinary skill in the art, "site-specific pegylation remains a chemical challenge" (see Veronese et al). What was the motivation therefore for combining Gaertner with Namiki? To increase bioavailability through attaching a large PEG group? This seems to be the asserted problem to be solved; and yet Gaertner itself teaches against this procedure working for sites other than serine and threonine and achieved other than by an oxime bond (for example, the site attachment of mPEG to the IL-8 protein is contrasted against the modifications needed for attachment involving amino groups, (pg 41) and sometimes necessitated even the removal of the N-terminal amino group (IL-1ra protein mPEgylation, pg 42). Additionally, Gaertner discloses that a 20kDa mPEGylation of G-CSF to the N-terminus resulted in a 2-3 fold **decrease** of activity. (pg 43). Finally and notably, Gaertner concludes that while its approach of oxime bond attachment via serine/threonine site for single site PEG attachment works for oxidized IL-8, other methods must rely on attachment of mPEG chains of relatively small size (5kD) to several amino groups of the protein. Indeed, the last line of Gaertner summarizes the whole essential teaching of Gaertner: "to investigate the attachment of even larger multibranched polymers to the single reactive site (serine or threonine) of the protein (IL-8) via an **oxime** bond." Or, as the Gaertner abstract succinctly notes: "***This method (large PEGylation of a specific site on a protein) relies on the ability to generate a reactive carbonyl group in place of the terminal amino group.....to form a stable oxime bond***."

The abstract of Gaertner therefore also teaches that the proteins of Gaertner are modified and changed to form an acceptable site for the bonding of the PEG. This is contrary to Claim 2 of Applicant's invention, which requires an amide (not oxime) bond. Additionally, Gaertner notes a 20kDa mPEGylation attachment to the G-CSF protein to the N-terminus resulted in a 2-3 fold **decrease** of biological activity. This disclosure

would teach away from the attachment of a 20 kDa (to 40 kDa) PEG group to the NK4 protein, as claimed in Applicant's claim 1. Applicant's invention was to, inter alia, improve the bioavailability of the resulting NK4-PEG conjugate. Accordingly, there would be little if any motivation to combine an attachment of such a size PEG, which resulted in a decrease of activity with regard to one protein, to another protein.

The Examiner finally, in the Advisory Action of March 24, 2006, seems to acknowledge that the obviousness rejection of the Office Actions relies on "trial and error" experimentation: "**Even if some pegylation were to decrease activity of the protein, it would have been obvious to experimentally determine which types of pegylation would result in a protein with improved characteristics.**" (Advisory Action, page 2). In other words, the obviousness rejection rests upon the presumption that it would be "obvious to try"! However, obviousness of a process must be predicated on something more than it would be obvious to try. Ex parte Agrabright et al., 161 U.S.P.Q. 703 (POBA 1967). There is usually an element of "obvious to try" in any research endeavor, since such research is not undertaken with complete blindness but with some semblance of a chance of success. "Obvious to try" is not a valid test of patentability. In re Mercier, 185 USPQ 774 (CCPA 1975). The current rejection that it would be "obvious to try" the PEG procedure of Gaertner to attach large PEG groups, which in Gaertner were attached to only specific proteins at one of two specific sites via an oxime bond, to a different protein disclosed by Namiki (claim 1) at a different site via a different (amide) bond (claim 2) does not meet the requirement of the statute (35 USC 103) that the issue of obviousness be based on the subject matter as a whole. In re Yater, 211 USPQ 1149 (CCPA 1981). This failure to meet the requirement of the statute and Yater is even more acute when the Gaertner disclosure that a 20kDa mPEGylation of G-CSF to the N-terminus resulted in a 2-3 fold **decrease** of activity is considered. As a whole, the subject matter taught that the pegylation of protein field to increase biological activity was unpredictable.

Therefore, "at the time the invention was made", the prior art was at best inconclusive and in reality contradictory with regard to which site specific sites should

be used for which proteins. Indeed, there seemed to be no general procedure or general result. Sometimes large PEG attachment worked to increase biological activity on a certain protein, and sometimes it decreased the biological activity of the exact same protein! There was simply no motivation to combine Gaertner and Namiki. On the contrary, Applicants submit that there was instead a motivation not to combine these references given the teachings of Gaertner, Francis, Reddy, Mehvar and Veronese. For example, why attach a 20 kDa PEG group to NK4 (Applicant's Claim 1) when Gaertner reports the a 20kDa mPEGylation of G-CSF resulted in a 2-3 fold **decrease** of biological activity? Even the reference adduced by the Examiner for the first time in the March, 2006 Advisory Action, for the proposition that "the skill of the art is high" actually supports Applicant's position that this art was far from settled and that Pegylation at different proteins at different sites (and different sized PEGs) was not obvious. See Lu et al references (*compare* the 1994 "Pegylated Peptides II" Int. J. Pept. Protein Res.(43) at 127 disclosing N-terminal pegylation, side-chain pegylation and C-terminal pegylation of IL-2 protein, each by different procedures and at different sites (See abstract) *with* the 1995 "Pegylated Peptides IV" Int. J. Pept. Protein Res.(46) at 253 disclosing reduced potency of pegylated acetylated N-terminal sites, regardless of PEG size, decreased potency of specific site pegylations, and increased potency of C-terminal pegylations, all involving the protein hGRF and concluding "the biological activity (of pegylated hGRF) is highly dependent on the site of pegylation and, in some cases, the molecular weight of PEG (degree/size of pegylation) moiety used" (See abstract)). In light of the Lu et al references, Gaertner, Reddy, Francis and Mehvar, one of ordinary skill in the art would have known only that the PEGylation of proteins to improve bioavailability is a non-obvious, unpredictable field that involves lots of "trial and error".

(b) There Is No Motivation To A Person Of Ordinary Skill In The Art

The Examiner relies on the capabilities of one of ordinary skill in the art to establish the requisite motivation to combine the references (and specifically Gaertner

with Namiki and Date). For example, in the Advisory Action of March 24, 2006 (page 2), the Examiner states:

> ...(i)t is also known in the art that some proteins may be suboptimally active when PEGylation is achieved at the N-terminus an(d) therefore, site specific PEGylation is well practiced. ... **Even if some pegylation were to decrease activity of the protein, it would have been obvious to experimentally determine which types of pegylation would result in a protein with improved characteristics.**

However, as stated above, the Federal Circuit has held that the level of skill in the art cannot be relied upon to provide the suggestion to modify or combine references. *Al-Site Corp. v. VSI Int'l Inc.*, 174 F3d 1308, 1324 (Fed. Cir. 1999) (the fact that a claimed invention is within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish obviousness).

Far from supporting the Examiner's position, the above cited Lu et al reference (entitled "Pegylated Peptides II") supports Applicant in that it teaches pegylation of the IL-2 protein at the N, COOH and side-chain positions together without recommending a particular PEG moiety and/or size. Moreover, one year after the publication of this article in a related follow-up research publication in the same journal, Lu et al showed that with regard to another protein, hGRF, that N-terminal PEGylation actually reduced activity (even regardless of the specific PEG site), while C-terminal PEGylation increased activity regardless of PEGylation size. See, attached Lu et al article (Pegylated Peptides IV, Int. J. Pept Protein Res.,1959(46) 253-264).

Even if there was motivation to combine Namiki and Gaertner, again no motivation exists to select just a portion of Namiki, as allegedly suggested by Date and modify it by the attachment of a large PEG of Gaertner to an attachment point that Gaertner specifically teaches away from. "[T]here is nothing in the disclosure of [Namiki and Gaertner] suggesting that one should select such variables." *In re Baird*, 16 F.3d at 383. Indeed, Gaertner teaches away from the amide bond of Namiki and

teaches that the proteins must be modified. As in the *In re Langer* case, there can be no motivation to select an isolated possibility of a structurally similar compound absent a showing that Namiki or Gaertner distinguishes such **isolated** possibilities of structurally similar compounds **as a class** from the plethora of compounds that are not structurally similar- which neither reference does. As stated above, Namiki teaches and requires small PEG at the amide terminus while Gaertner teaches large PEGylation molecules and requires same at *oxime bond site(s).* In addition, Gaertner discloses that a 20 kDa mPEGylation of G-CSF to the N-terminus resulted in a 2-3 fold **decrease** of activity. *In contrast, the present invention requires a large PEG (20 to 40 kDa) attachment on the NK4 protein (Claim 1) at an amide bond site (Claim 2)..* Even if there was motivation to combine Namiki with Gaertner, some additional motivation or suggestion would be necessary to 1) suggest such a large PEG attachment to the NK4 protein to overcome Gaertner's disclosure of the decrease of activity (with regard to Applicant's Claim 1), and 2) suggest replacement oxime bonding site with the amide bonding site to overcome Gaertner's teachings away from such a combination (with regard to Applicant's Claim 2). These additional motivations do not exist – indeed there is no motivation adduced to even attach a large PEG at the N-terminus of NK4 (as in Applicant's Claim 1), especially in light of the 20 kDa PEG attachment to G-CSF decreasing activity. Motivation to combine the references to reach Applicant's claimed invention in Claim 1, its dependents thereto and claim 2 is lacking.

The Patent Office's approach to selectively combining just one part of Gaertner to a part of Namiki to arrive at Applicant's claimed invention, *while ignoring unhelpful sections of the references*, is contrary to well established legal precedent. Note, for example, the CCPA's admonition in reversing the Patent Office's obviousness rejection in *In re Mercier*, 185 USPQ 774, 778 (CCPA 1975):

> These and other questions arise because the board's approach fails to recognize that all of the relevant teachings of the cited references must be considered in determining what they fairly teach to one having ordinary skill in the art ...

* * *

> The relevant portions of a reference include not only those teachings which would suggest particular aspects of an invention to one having ordinary skill in the art, but also those teachings which would lead such a person away from the claimed invention. [Citations omitted; emphasis in original].

In summary: (1) there is no structural similarity between any compound disclosed in Gaertner or Namiki and the claimed invention for the reasons stated above; (2) even if the compounds identified by the Examiner in Namiki or Gaertner could be considered structurally similar, no motivation exists to select such compounds and then modify them to arrive at the claimed invention for the reasons stated above; and (3) furthermore, there is a lack of motivation to combine Namiki with Gaertner for the reasons stated above.

The cited references thus do not disclose or suggest a conjugate consisting of a NK4 molecule and a polyethylene glycol group having a molecular weight of about 20-40 kDa (Claim 1). They also do not disclose or suggest the polyethylene glycol group of: (i) the formula $-CO-(CH_2)_x-(OCH_2CH_2)_mOr$, (ii) is monomethoxy polyethylene glycol and (iii) forms amide group with the amino groups of N-terminal NK4 fragment (Claim 2). In contrast, the cited references teach away from Applicants invention and cannot be properly combined.

Accordingly, for all of the above reasons, the Applicants respectfully submit that claims 1-2, 4-6, 8 and 12-13 are not obvious under 35 U.S.C. § 103; and respectfully request that the Board direct the Examiner to allow such claims.

2. Obviousness Rejection Under 35 USC §103(a)

A. Claims 14 and 15

(1) The Examiner's Rejection

The Examiner has rejected claims 14 and 15 under 35 USC 103(a) as being unpatentable over Namiki et al (EP 0816381 A1, published on 07/01/1998) in view of Date et al (Oncogene, 17:3045-3054, 1998) and Gaertner et al (Bioconjugate 7:38-42, 1996) and further in view of Veronese (US Patent 6,528,485 B1). The Examiner asserts that since Veronese et al disclose making PEGylated proteins and purifying them to greater than 92% purity, Veronese would teach a higher purity PEGylated protein resulting in a better bioavailability and pharmacokinetics in vivo.

(2) Applicant's Arguments in Response

The addition of the Veronese reference to the above-described references does not overcome the deficiencies of said references and this does not render Applicants' invention obvious. Veronese concerns the pegylation of HGRF only. It does not address pegylation at any other protein (much less pegylation of NK4) and requires unique coupling conditions optimized for hGRF wherein, in contrast to the current invention, the PEG is coupled to the protein via a norleucine or lysine linker and not via a monomethoxy linker. In fact, Veronese only claims a peg-hgf complex that does not contain a triazine group. Moreover, and in support of Applicants' invention, Veronese specifically admits that "site-specific pegylation remains a chemical challenge." Finally, there is no motivation to combine Veronese with Gaertner, as Gaertner's specification requires the sides to be serine or threonine only.

As Veronese does not supply any motivation to combine the prior references, nor provide any details for preparing the PEGylated compounds of the present claimed invention and - indeed – admits the specific challenges associated with PEGylation,

Applicants respectfully suggest that claims 14 and 15 are thus not obvious for the reasons stated above in Section VII Argument, Part 1.

VIII. CONCLUSION

In summary, the current invention describes a conjugate of NK4 with a 20 to 40 kDa PEG (Claim 1). The invention also describes the PEGylation of an N-terminal amino acid of NK4 via amide bond formation between an N-terminal amino group and a carboxyl group of a functionalized monomethoxy-PEG, with the molecular weight of the attached linear PEG is of between 20 kDa and 40 kDa (Claim 2). The cited references do not singly or in combination disclose or suggest a conjugate consisting of a NK4 molecule and a polyethylene glycol group having a molecular weight of about 20-40 kDa (Claim 1), nor further do the references disclose or suggest the polyethylene glycol group of: (i) the formula $-CO-(CH_2)_x-(OCH_2CH_2)_mOr$, (ii) is monomethoxy polyethylene glycol and (iii) forms amide group with the amino groups of N-terminal NK4 fragment (Claim 2). In contrast, the cited references teach away from Applicants invention and cannot be properly combined.

In view of the foregoing arguments and the facts of record, the Appellants' respectfully submit that claims 1-2, 4-6, 8 and 12-15 are in condition for allowance and respectfully request that the Board direct the Examiner to allow such claims.

An oral hearing is desired and will be formally requested at the appropriate time pursuant to 37 C.F.R. § 41.47(b). No other fee is believed to be due in connection with the filing of this Appeal Brief other than the fees noted above and submitted concurrently herewith. However, if any other fee is deemed necessary, authorization is given to charge the amount of any such fee to Deposit Account No. 08-2525.

Respectfully submitted,

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233916

In the Claims:

1. (Original) A conjugate consisting of a NK4 molecule and a polyethylene glycol group having a molecular weight of from about 20 to about 40 kDa.

2. (Original) The conjugate according to claim 1, wherein said polyethylene glycol group has the formula

$$-CO-(CH_2)_X-(OCH_2CH_2)_mOR$$

and said –CO group forms an amide bond with one of the amino groups of the N-terminal fragment of said NK4, wherein

X is 2 or 3;

m is from about 450 to about 950;

R is $(C_1\text{-}C_6)$alkyl.

4. (Original) The conjugate according to claim 1, wherein the polyethylene glycol group has a molecular weight of from about 30 to about 40 kDa.

5. (Original) The conjugate according to claim 1, wherein said polyethylene glycol group is selected from monomethoxy polyethylene glycol groups.

6. (Original) The conjugate according to claim 1, wherein the polyethylene glycol group is selected from the group of linear PEG chains and branched PEG chains.

8. (Original) The conjugate according to claim 1, wherein the polyethylene glycol group is attached to a group selected from the lysine side chains and the N-terminal amino group of the NK4 molecule.

12. (Original)A pharmaceutical composition comprising conjugates of claim 1 and at least one pharmaceutically acceptable carrier.

13. (Original) A composition comprising conjugates of NK4 monoPEGylated with polyethylene glycol groups that have a molecular weight of from about 20 to about 40 kDa, wherein the conjugates comprise conjugates in which the polyethylene glycol groups are attached to groups selected from the lysine side chains of NK4 molecules and conjugates in which the polyethylene glycol groups are attached to the N-terminal amino groups of NK4 molecules.

14. (Original) A composition comprising conjugates of NK4 monoPEGylated with polyethylene glycol groups that have a molecular weight of from about 20 to about 40 KDa, wherein said monoPEGylated conjugates comprise at least 90% of the total of pegylated NK4 molecules and unpegylated NK4 molecules in the composition.

15. (Original) The composition according to claim 14, wherein said monoPEGylated conjugates comprise at least 92% of the total of pegylated NK4 molecules and unpegylated NK4 molecules in the composition.

EVIDENCE APPENDIX

1. Namiki et al -EP 0816381 A1, (07/01/1998)
2. Date et al, Oncogene, 17:3045-3054, (1998)
3. Gaertner et al, Bioconjugate 7:38-42, (1996)
4. Veronese - US patent 6,528,485 B1
5. Lu et al (Pegylated Peptides IV, Int. J. Pept Protein Res.,1959(46) 253-264).
6. Lu et al (1994 "Pegylated Peptides II" Int. J. Pept. Protein Res.(43)
7. Francis, G.E., et al., International Journal of Hematology, Vol. 68 (1998) pgs. 1-18
8. Reddy, R., et al. Annals of Pharmacotherapy Vol. 34, No. 7-8 (7/2000) pgs. 915-923
9. Mehvar, R.J. Pharm. Pharm. Sci. 3(1) (2000) pgs. 125-136
10. Office Action - September 2, 2005
11. Advisory Action - March 24, 2006

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(54) PEG-MODIFIED HGF

(57) This invention relates to a PEG-modified HGF (Hepatocyte Growth Factor), namely HGF modified by polyethylene glycol. The PEG-modified HGF of the invention has a prolonged clearance in vivo, effectively exhibits its physiological activity for a long period of time, and has the same physiological activity as HGF, which makes it possible to reduce the dose and relieve the side effects of the same.

HGF = SF ~ NK4

EP 0 816 381 A1

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Description

Field of the invention

This invention relates to a PEG-modified HGF, namely Hepatocyte Growth Factor (HGF) modified by an agent comprising a Polyethylene Glycol (PEG) moiety. This invention also relates to a pharmaceutical composition comprising administering to a patient an effective amount of PEG-modified HGF, for treating hepatic diseases, treating renal diseases, promoting growth of epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cells, restoring hair, as a component of skin cosmetics.

Background of the invention

HGF is a unique cytokine having various activities not limited to controlling growth of hepatocytes, and growth of various epithelium cells, but also extended to enhancing motility of cells, and inducing morphogenesis with constructing three dimensional tissue. HGF takes a main role in generation or regeneration of tissues and organs as a mitogen, a motogen and a morphogen.

HGF is a heterodimeric protein comprising an α chain having a molecular weight of about 69 kD and a β chain having a molecular weight of about 34 kD, and having a molecular weight 82-85 kD as a whole.

HGF acts as a growth factor for promoting growth of not only hepatocytes, but also renal tubule epithelial cells, keratinocytes, melanocytes, alveolar epithelial cells type II, gastric mucosal epithelial cells, vascular endthelial cells, and other epithelial cells.

HGF shows an activity for promoting growth of normal cells, but shows an activity for suppressing the proliferation of tumor cells (Tajima, H. et al., FEBS Lett., 291, 229, 1991).

HGF also shows an activity for enhancing motility of various epithelial cells such as MDCK cells (normal epithelial cells of renal tubule) and a motogen activity such as scattering colony of cultured said cells.

HGF is reported to have an activity of inducing morphogenesis of MDCK cells (Montesano, R. et al., Cell, 67, 901, 1991).

HGF inhibits leakage of a soluble enzyme from cultured hepatocytes prepared from an animal treated by carbon tetrachloride, a typical toxic substance to liver. These results show that HGF has an anti-hepatitis activity in vitro. HGF is also reported to have an anti-hepatitis activity in vivo (Takehara, T. et al., Biomed. Res., 12, 335, 1991).

HGF is also reported to enhance the activity of Na-K-ATPase in renal tubule epithelial cells and has been suggested to enhance renal functions (Ishibashi, T et al., Biochem. Biophys. Res. Commun., 182, 960, 1992).

Problems in administering HGF in vivo are fast clearance in vivo and that HGF originating from non-human species may have an antigenicity.

One of the effective ways to solve the problems is modifying the protein with a PEG reagent to delay the clearance and to reduce the antigenicity (Yoshimoto, T. et al., Jpn. J. Cancer Res., 77, 1264, 1986; Japanese patent application KOKAI 56-23587, 1981; Japanese patent application KOKAI 61-178926, 1986; Abuchowski, A. et al., Cancer Biochem. Biophys., 7, 175, 1984; Japanese patent application KOKAI 62-115280, 1987).

As described above, HGF has various biological activities and development of HGF derivatives as pharmaceutical agents is expected. It is considered important to develop a pharmaceutical composition for use in a method of treatment, comprising administration to a patient an effective amount of HGF for treating hepatic diseases, treating renal diseases, promoting growth epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cells, restoring hair, and in skin cosmetics, but it is necessary to administer several ten μg/kg to several mg/kg of HGF to have enough activities, and the administration amount may be relatively higher than other physiologically active protein. High dose may be disadvantageous because of unexpected side effects and high cost to manufacture a pharmaceutical product.

The most important problem to be solved is to improve the clearance in vivo, since it is reported that half life time of HGF in blood is quite short (α phase: about 2 minutes, β phase: about 20 minutes). But a pharmaceutical composition for use in a method comprising administration of an effective amount of PEG-modified HGF having improved pharmacokinetics in vivo and improved activities to a patient for treating hepatic diseases, treating renal diseases, promoting growth epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cells, restoring hair, and in skin cosmetics, has not been reported.

The subject of the present invention is to provide a PEG-modified HGF that can maintain bioactivities in vivo for a longer time to reduce the amount of administration, and has a specificity to targeting organ.

Disclosure of the invention

As a result of extensive study, the present inventors have succeeded in obtaining a PEG-modified HGF that has improved properties in vivo and prolonged HGF activities.

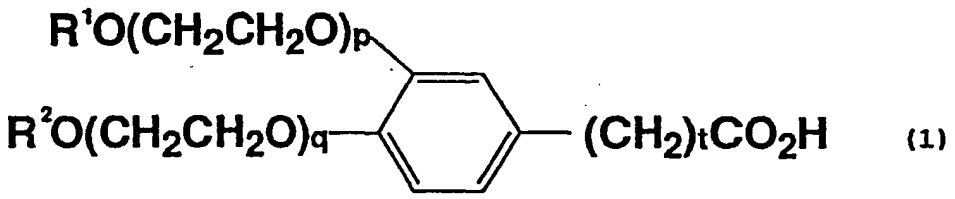
The present invention provides a PEG-modified HGF.

The present invention also provides;

a PEG-modified HGF comprising HGF modified by a PEG reagent that modifies an amino group of lysine or N-terminal of a protein;

a PEG-modified HGF comprising HGF modified by a PEG reagent that modifies an amino group of lysine or N-terminal of a protein linked through an amide bond;

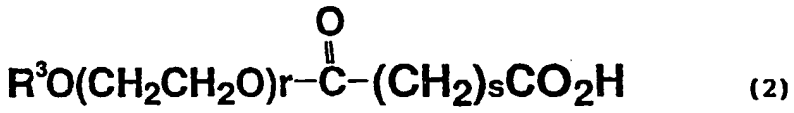
a PEG-modified HGF which is obtained by a process comprising the steps of, activating a carboxyl group of a PEG agent represented by the formula (1):



(1)

(wherein R^1 and R^2 are the same or different and independently a lower alkyl; p and q are the same or different and independently an integer selected from the range of from 20 to 280; and t is 0 or a positive integer), and treating said activated reagent with HGF; and

a PEG-modified HGF which is obtained by a process comprising the steps of, activating a carboxyl group of a PEG agent represented by the formula (2):



(2)

(wherein R^3 is a lower alkyl, r is an integer selected from the range of from 20 to 280, and s is a positive integer), and treating said activated reagent with HGF.

Present invention also provides a pharmaceutical composition for use in a method comprising administration of an effective amount of a PEG-modified HGF to a patient for treating hepatic diseases, treating renal diseases, promoting growth epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cell, restoring hair, and in skin cosmetics.

The present invention also provides a method of using a PEG-modified HGF comprising the step of manufacturing a pharmaceutical composition for treating hepatic diseases, treating renal diseases, promoting growth epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cells, restoring hair, and in skin cosmetics.

Brief description of drawings

Figure 1 shows hepatocyte growth activity of a PEG-modified HGF.

Figure 2 shows time dependent concentration of a PEG-modified HGF in blood.

Figure 3 shows an enhancing activity of a PEG-modified HGF on production of protein (fibrinogen) .

Best mode to carry out the present invention

In the present invention, HGF prepared by various methods can be used.

The methods of preparing HGF are well known to a person skilled in the art. For example, HGF may be prepared by a process comprising the steps of;

extracting from an organ (such as liver, spleen, lung, bone marrow, brain, kidney, placenta and the like), blood cells (such as platelets, white blood cells and the like), plasma, serum and the like, of a mammal (such as rat, bovine, horse, sheep and the like); and purifying (FEBS Letters, 224, 312, 1987; Proc. Natl. Acad. Sci. USA, 86, 5844, 1989, etc.).

HGF may also be prepared by a process comprising the steps of;

culturing cells in primary culture or a cell line which produce(s) HGF; extracting from the culture products (supernatant fluid, cultured cells, etc.); and purifying.

HGF may also be prepared by a genetic engineering method comprising the steps of;

inserting a gene encoding HGF to an appropriate vector;
transfecting a host cell by inserting said inserted vector; and purifying from the supernatant fluid of the cultured transfected cells (for example, Nature, 342, 440, 1989;
Japanese patent application KOKAI 5-111383 (1993); Japanese patent application KOKAI 3-255096 (1991); Biochem. Biophys. Res. Commun., 163, 967, 1989).

Said host cell is not limited, and various host cells conventionally used in genetic engineering methods can be used, which are, for example, Escherichia coli, Bacillus subtilis, yeast, mold fungi, plant or animal cells and the like.

A more specific process of preparing HGF from a living tissue comprises the steps of;

administering carbon tetrachloride to a rat intraperitoneally to make said rat hepatitis;
removing a liver from said rat and homogenizing; and purifying by a conventional method of protein purification such as gel column chromatography (such as S-Sepharose, heparinsepharose and the like), HPLC and the like.

HGF may be prepared by a genetic engineering process comprising the steps of;

transforming an animal cell (such Chinese Hamster Ovary (CHO) cells, mouse C127 cells, monkey COS cells, Sf (Spodoptera frugiperda) cells and the like) with a gene encoding amino acid sequence of HGF; and
purifying from the supernatant fluid of the cultured cells.

HGF includes human HGF and mammalian HGF, preferred HGF is a human HGF, and more preferred HGF is a human recombinant HGF (Japanese patent application KOKAI 5-111383 (1993)).

HGF prepared by the above processes includes any HGF that has substantially the same activities such as a partial deletion derivative of the amino acid sequence, a substitution derivative of an amino acid, an insertion derivative of other amino acid sequence, a derivative from binding one or more amino acids to N- or C-terminus of the amino acid sequence, or sugar chain deletion or substitution derivatives.

In the present specification, "PEG-modified" includes modifying a protein with a PEG reagent.

A PEG reagent means having a portion of a polyethylene group; $-O-(CH_2CH_2O)n-$ (wherein n is an integer selected from the range of from 20 to 280) and a portion to be able to bind to a protein.

There are many PEG reagents that may be used in the invention and examples of preferred PEG reagent include the three types described below.

1. A PEG reagent modifying an amino group of lysine or the N-terminus of a protein

A PEG reagent modifying an amino group of lysine or the N-terminus of a protein comprises a reagent having a group that can bind to a protein, such as a carboxyl group, a derivative of carboxyl group, a carbonate ester group, formic group and the like. Examples of the PEG reagent modifying an amino group of lysine or the N-terminus of a protein are described in the reports cited below.

① Reagent modifying HGF through an amide bond

1) Tetrahedron, 40, 1581 (1961).

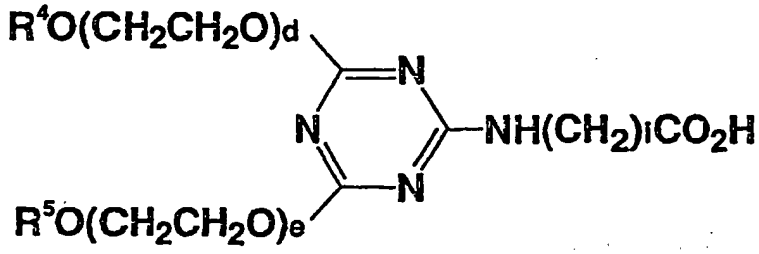
2) Anal. Biochem., 131, 25 (1983)
3) Cancer Biochem. Biophys., 7, 175 (1984)
4) Proc. Natl. Acad. Sci. USA, 84, 1487 (1987)
5) FEBS Letters, 223, 361 (1987)
6) Japanese patent application KOKAI 61-249388 (1986)
7) Japanese patent application KOKAI 1-316400 (1989)
8) Japanese patent application KOKAI 4-108827 (1992)

The reagents described in the reports are represented below;

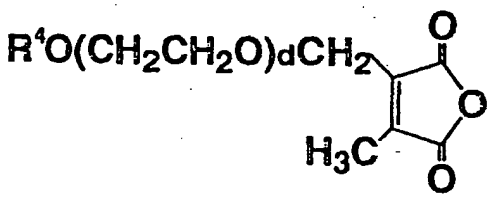
$R^4O(CH_2CH_2O)d\text{-}CO_2H$

$R^4O(CH_2CH_2O)d\text{-}CH_2CO_2H$

$R^4O(CH_2CH_2O)d\text{-}CH_2CH_2NHCO(CH_2)iCO_2H$



$R^4O(CH_2CH_2O)d\text{-}CH_2CHO$



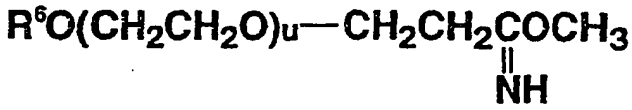
$HOCOCH_2O(CH_2CH_2O)dCH_2CO_2H$ (wherein R^4 and R^5 are same or different and independently a lower alkyl, d and e are the same or different and independently an integer selected from the range of from 20 to 280, and i is a positive integer).

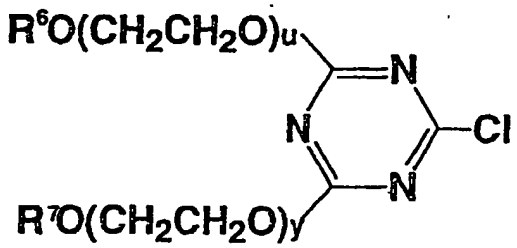
With these above reagents, the preferred range of i is from 1 to 10, and more the preferred range of i is from 1 to 4.

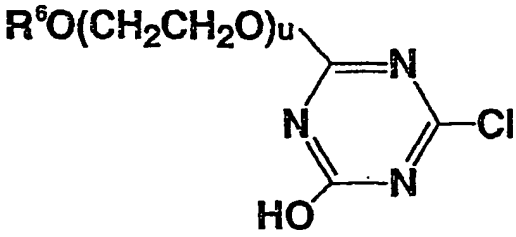
② A reagent modifying HGF through a bond other than ①

1) J. Biol. Chem., 257, 3578 (1977)
2) Chem. Lett., 773 (1980)
3) Res. Commun. Chem. Pathol. Pharmacol., 29, 113 (1980)
4) Agr. Biol. Chem., 52, 1575 (1988)
5) J. Biomatter. Sci. Polymer, Edn., 2, 61 (1991)
6) Japanese patent application KOKAI 61-178926 (1986)
7) Japanese patent application KOKAI 53-10800 (1988)

The reagents described in the reports are represented below;







(wherein R^6 and R^7 are the same or different and independently a lower alkyl, and u and y are the same or different and independently an integer selected from the range of from 20 to 280).

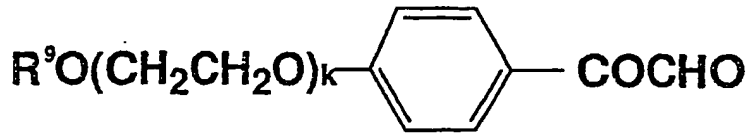
2. PEG reagent modifying a carboxyl group of aspartic acid, glutamic acid or the C-terminus of a protein

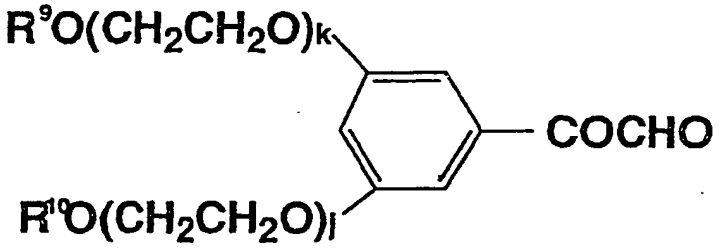
A PEG reagent modifying a carboxyl group of aspartic acid, glutamic acid or the C-terminus of a protein comprises a reagent having a group that can bind to a protein, such as an amino group and the like. Examples of the PEG reagent are described in Japanese patent application KOKAI 56-23587 (1981) and the like;

$R^8O(CH_2CH_2O)w\text{-}CH_2CH_2NH_2$ (wherein R^8 is a lower alkyl, and w is an integer selected from the range of from 20 to 280).

3. PEG reagent modifying a guanidino group of arginine

A PEG reagent modifying a guanidino group of arginine comprises a reagent having a group that can bind to a polypeptide or protein, such as phenylglyoxal and the like. Examples of the PEG reagent are a reagent described in Japanese patent application KOKAI 2-117920 (1990) or Japanese patent application KOKAI 3-88822 (1991);





(wherein R^9 and R^{10} are the same or different and independently a lower alkyl, and k and j are the same or different and independently an integer selected from the range of from 20 to 280).

Preferred examples of the PEG reagent are a PEG reagent modifying an amino group of lysine or the N-terminus of a protein and the like. More preferred examples of the PEG reagent are a PEG reagent modifying an amino group of lysine or the N-terminus of a protein through an amide bond.

The PEG reagent may be prepared by a conventional method well known to the skilled in the art of organic chemistry. For example, the PEG reagents may be prepared by the methods described in the above prior art.

The preparation of the PEG-modified HGF using the PEG reagent can be carried out by a conventional method well known to the skilled in the art of organic chemistry. For example, the PEG-modified HGF may be prepared by the method described in the above prior art.

A detailed method is exemplified below using a PEG reagent that has a carboxyl group in a molecule.

The PEG modification may be accomplished by the two-step reactions described below.

1. Activating a carboxyl group of the PEG reagent

Examples of methods of activating a carboxyl group are an activated ester method, a mixed anhydride method, and the like. Methods of activating a carboxyl group are described in Seikagaku Jikken Kouza Vol 1, Tanpakushitu No Kagaku IV 236-242 (Tokyo Kagaku Dojin) and Peptide Gousei No Kiso To Jikken (Izumiya et al., Maruzen).

Examples of the activated ester are p-nitrophenyl ester; thiophenyl ester; p-nitrothiophenyl ester; 1,3,5-trichlorophenyl ester; pentachlorophenyl ester; pentafluorophenyl ester; 2,4-dinitrophenyl ester; cyanomethyl ester; dicarbonicimide ester such as N-hydroxyphthalimide ester, N-hydroxysuccinimide ester and the like; activated hydroxylamine ester such as N-hydroxypeperidine ester, N-hydroxy-5-norbornene-2,3-dicarbonic acid ester and the like; and the like.

The activated ester may be prepared by a conventional organic chemistry preparation method of ester, such as treating a carboxyl group of the PEG reagent with an alcohol derivative corresponding to the activated ester in the presence of a condensing reagent such as dicyclohexyl-carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and the like at a temperature ranging from -20°C to room temperature for from 1 to 24 hours.

The activated ester may also be prepared by treating a carboxyl group of the PEG reagent with a halogenated derivative corresponding to the activated ester in the presence of a base such as triethylamine and the like at a temperature ranging from 0°C to 80°C for from 1 to 72 hours.

The activated ester prepared by the method described above may be used in the next reaction immediately after working up, when the activated ester is unstable. The stable activated ester may be isolated and may be stored for a long time.

The mixed anhydride may be prepared by treating a carboxyl group of the PEG reagent with iso-butylchloroformate, ethylchloroformate, pivaloyl chloride, isovalerile chloride, diphenylphosphyl chloride and the like in the presence of base such as N-methylmorphorine, N-ethylpiperidine and the like at a temperature ranging from -20°C to 0°C for from 1 to 30

minutes. The mixed anhydride obtained in above method may be used in next reaction without isolation or purification.

2. Treating HGF with the PEG reagent having an activated carboxyl group

The reaction of a PEG reagent having an activated carboxyl group and HGF may be carried out at a temperature selected from the range such that activity of HGF can be maintained. A preferred temperature range is from 0 to 25°C.

A molar amount of the PEG reagent may be selected from the range of from 5 to 100 times of the molar of HGF. In the case of modifying an amino group of lysine or the N-terminus of protein, a preferred molar range of the PEG reagent is from 10 to 25 times of the molar of HGF.

Since the PEG reagent represented by the formulae (1) or (2) that can be used in the invention may be treated in the reaction at a pH of more than 5.5, the pH of the modifying reaction can be selected from a pH range of more than 5.5, where HGF is not deactivated. A preferred pH of the modifying reaction may be selected from the range of from 7 to 9.

A reaction solvent may be selected from solvents that are inert to the reaction. Examples of the reaction solvent are a buffer solution such as phosphate buffer solution, tris buffer solution, borate buffer solution, aqueous sodium carbonate solution, aqueous sodium hydrogencarbonate solution, N-ethylmorpholine-acetic acid buffer solution, sodium maleate buffer solution, sodium acetate buffer solution and the like.

An organic solvent that does not deactivate HGF and is inert to the reaction may be added to the solvent, and examples of such organic solvent are a lower alcohol such as methanol, ethanol, propanol and the like; acetonitrile; dioxane; tetrahydrofuran; and the like.

The reaction time is selected from the range of from 1 to 72 hours.

After the termination of the reaction, the PEG-modified HGF may be purified from the reaction mixture by a conventional purification method of a protein, such as salting-out, gel filtration, ion-exchange chromatography, adsorptive chromatography, affinity chromatography, ultrafiltration, reverse phase HPLC and the like.

The lower alkyl as used in R^1-R^{10} of the PEG reagent in the invention includes a straight and branched alkyl having 1 to 6 carbon atoms. Examples of lower alkyl are methyl, ethyl, propyl, iso-propyl, butyl, pentyl, hexyl and the like.

The PEG-modified HGF of the present invention may be formulated in various ways such as liquid preparations, solid preparations, capsule preparations and the like. The PEG-modified HGF of the present invention may be formulated for parenteral administration for injection without any carrier or with an appropriate conventional carrier and for oral administration with an appropriate conventional carrier. The preparation for parenteral administration for injection may be prepared by a conventional method, such as a method comprising the steps of;

dissolving the PEG-modified HGF in an appropriate solvent such as sterilized water, buffered solution, physiological saline solution and the like;
sterilizing by filtration; and
filling a sterilized bottle with said solution.

An amount of the PEG-modified HGF in the parenteral preparation is from about 0.0002 to about 0.2 (W/V%), and preferably from about 0.001 to about 0.1 (W/V%). The preparation may be prepared by the conventional preparation technique. The amount of the PEG-modified HGF may be varied depending on preparation form, disease to be treated and the like.

A stabilizer may be added to the preparation, and examples of the stabilizer are albumin, globulin, gelatin, mannitol, glucose, dextran, ethylene glycol and the like. The preparation of the present invention may include a necessary additive such as an excipient, a solubilizing agent, an antioxidant agent, a pain-alleviating agent, an isotonic agent and the like. In the liquid preparation, it is preferable to store it under frozen condition or after removal of water by a process such as freeze-drying. The freeze-dried preparation is used by dissolving again in distilled water for injection and the like before use.

An administration route of the preparation of the present invention may vary depending on the form of preparation. The parenteral preparation may be administered intravenously, intraarterially, subcutaneously or intramuscularly. The amount of dose may vary depending on symptom, age, body weight, etc of a patient. The dose of PEG-modified HGF is calculated as the HGF portion of PEG-modified HGF and is selected from the range of from 0.1μg/kg to 10mg/kg. The preparation of PEG-modified HGF may be administered once or several times per day.

The utility of the present invention

The PEG-modified HGF of the present invention is considered to act on cells in the same mechanism as HGF, through the protein portion of the PEG-modified HGF. The PEG-modified HGF has same biological activities as HGF.

The HGF modified by the PEG reagent represented by the formulae (1) and (2) has an improved pharmacokinetic

activity in vivo, and more specificity to an organ compared to the non-modified HGF and shows same activities in lower dose.

The PEG-modified HGF has a prolonged clearance in vivo and shows retained biological activities. The PEG-modified HGF has the same activities as non-modified HGF. Therefore the PEG-modified HGF is useful as a pharmaceutical composition for treating hepatic diseases, treating renal diseases, promoting growth of epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cell, restoring hair, and in skin cosmetics.

Examples

The following examples are for illustrative purposes only and are not to be construed as limiting the invention.

The data of amino acid analysis in the examples are the results in the acid decomposition of the modified HGF (decomposition products after treating with 6 N hydrochloric acid-phenol at 110 °C for 24 hours).

The rate of modified amino group is calculated from the method of trinitrobenzene sulfonic acid (Methods in Enzymology, vol.25, p464 (1972). Academic Press, New York) comparing to non-modified HGF.

Each abbreviation means the following respectively.

Asx:	Aspartic acid or Asparagine
Glx:	Glutamic acid or Glutamine
Ser:	Serine
Gly	Glycine
His:	Histidine
Arg:	Arginine
Thr:	Threonine
Ala:	Alanine
Pro:	Proline
Tyr:	Tyrosine
Val:	Valine
Met:	Methionine
Ile:	Isoleucine
Leu:	Leucine
Phe:	Phenylalanine
Lys:	Lysine

Example 1

HGF modified by 3,4-bis-methoxypolyethyleneglycolhydrocinnamic acid

To a 30mM phosphate buffer solution (pH 6.0, 0.3M-NaCl) (6.9ml) was added HGF (3.90mg) and the pH was adjusted to 8.25 with 1N NaOH solution. To the solution was added 3,4-bis-methoxypolyethyleneglycolhydrocinnamic acid N-hydroxysuccinimide ester (average molecular weight is about 10,000) (3.14mg, 0.15 equivalent to the amino groups) and the reaction mixture was stirred for 18 hours at room temperature. The pH of the reaction mixture was adjusted to 6.5 with 0.1 N HCl and the reaction mixture was purified by gel filtration chromatography (Column: Sephacryl S-200HR 2.6 cmØ x 90 cm, Eluent: 0.2M aqueous NaCl solution. Flow rate: 1.4ml/min., Detection wave length: 220nm). The objective fractions were collected and subjected to desalting and concentrating by ultrafiltration with YM-15 membrane (Amicon) to give an aqueous solution (750ml) of titled compound (content of protein: 3.713mg/ml, modifying rate of amino acid; 19%).

Amino acid analysis

Asx 63.2 (83), Glx 50.5 (59), Ser 30.1 (38), Gly 54.0 (57), His 20.2 (22), Arg 33.3 (41), Thr 31.9 (38), Ala* 21.0 (21), Pro 35.0 (44), Tyr 24.4 (32), Val 26.5 (33), Met 11.4 (15), Ile 32.1(37), Leu 35.5 (37), Phe 16.0 (17), Lys 41.4 (41), Cys - (40)

*: standard amino acid, (): calculated data, -: data not measured

GPC

Column: TSK gel G3000SW 7.5 mmØ x 600 mm (manufactured by Toso Co., Ltd.)
Eluent: 10mM Tris buffer, 0.2M aqueous NaCl solution, 0.05%SDS
Flow rate: 0.6ml/min.
Detection wave length: 220 nm
Retention time: 18.517 min.

Example 2

HGF modified by 3,4-bis-methoxypolyethyleneglycolhydrocinnamic acid

To a 30mM phosphate buffer solution (pH 6.0, 0.3M-NaCl) (6.9ml) was added HGF (3.90mg) and the pH was adjusted to 8.25 with 1N NaOH solution. To the solution was added 3,4-bis-methoxypolyethyleneglycolhydrocinnamic acid N-hydroxysuccinimide ester (average molecular weight is about 10,000) (6.29mg, 0.3 equivalents to the amino groups) and the reaction mixture was stirred for 18 hours at room temperature. The pH of the reaction mixture was adjusted to 6.5 with 0.1N HCl and the reaction mixture was purified by gel filtration chromatography (Column: Sephacryl S-200HR 2.6 cmØ x 90 cm, Eluent: 0.2M aqueous NaCl solution, Flow rate: 1.4ml/min., Detection wave length: 220nm). The objective fractions were collected and subjected to desalting and concentrating by ultrafiltration with YM-15 membrane (Amicon) to give an aqueous solution (1.00 ml) of titled compound (content of protein: 3.722mg/ml, modifying rate of the amino groups; 32%).

Amino acid analysis

Asx 64.4 (83), Glx 52.2 (59), Ser 30.6 (38), Gly 54.8 (57), His 20.2 (22), Arg 33.7 (41), Thr 32.5 (38), Ala* 21.0 (21), Pro 35.4 (44), Tyr 25.2 (32), Val 26.8 (33), Met 12.5 (15), Ile 32.8 (37), Leu 35.7 (37), Phe 16.2 (17), Lys 41.9 (41), Cys - (40)

GPC

Column: TSK gel G3000SW 7.5 mmØ x 600 mm (manufacture by Toso Co., Ltd.)
Eluent: 10mM Tris buffer, 0.2M aqueous NaCl solution, 0.05%SDS
Flow rate: 0.6ml/min.
Detection wave length: 220 nm
Retention time: 18.183 min.

Example 3

HGF modified by monomethoxypolyethylenglycolsuccinic acid

To a 30mM phosphate buffer solution (pH 6.0, 0.3M-NaCl) (6.85ml) was added HGF (3.87mg) and the pH was adjusted to 8.24 with 1N NaOH solution. To the solution was added monomethoxypolyethyleneglycolsuccinic acid N-hydroxy-succinimide ester (average molecular weight is about 5,000) (3.14mg, 0.3 equivalents to the amino groups) and the reaction mixture was stirred for one hour at room temperature. The pH of the reaction mixture was adjusted to 6.5 with 0.1 N HCl and the reaction mixture was purified by gel filtration chromatography (Column: Sephacryl S-200HR 2.6 cmØ x 90 cm, Eluent: 0.2M aqueous NaCl solution, Flow rate: 1.4ml/min., Detection wave length: 220nm). The objective fractions were collected and subjected to desalting and concentrating by ultrafiltration with YM-15 membrane (Amicon) to give an aqueous solution (1.60 ml) of titled compound (content of protein: 1.813mg/ml, modifying rate of the amino groups; 33%).

Amino acid analysis

Asx 62.5 (83), Glx 49.8 (59), Ser 31.0 (38), Gly 55.1 (57), His 19.4 (22), Arg 33.3 (41), Thr 31.8 (38), Ala* 21.0 (21), Pro 35.5 (44), Tyr 23.9 (32), Val 26.9 (33), Met 13.0 (15), Ile 31.9 (37), Leu 35.8 (37), Phe 16.1 (17), Lys 40.9 (41), Cys - (40)

*: standard amino acid, (): calculated data, -: data not measured
*: standard amino acid, (): calculated data, -: data not measured

GPC

Column: TSK gel G3000SW 7.5 mmØ x 600 mm (manufacture by Toso Co., Ltd.)
Eluent: 10mM Tris buffer, 0.2M aqueous NaCl solution, 0.05%SDS
Flow rate: 0.6ml/min.
Detection wave length: 220 nm
Retention time: 18.225 min.

Example 4

The activities in vitro of PEG-modified HGF's obtained in Examples 1, 2, and 3 were measured with hepatocyte primary cultures. The hepatocytes were obtained from Wister rats (Male, 8 to 10-week old) and were cultured.

The rat hepatocytes were isolated and cultured according to the method by Nakamura (Shodai-baiyou-kansaiboujikken-hou, Gakkai-shuppan Center (1989)). Isolated hepatocytes were cultured for 24 hours in a medium comprising William's E (WE) medium containing 5% FCS, and were cultured in the serum-free medium containing various concentrations of the sample for 20 hours, in wells.

About 2 kBq/well of 5-[^{125}I]-iododeoxyuridine (^{125}I-Urd) was added to each well, and the cells were cultured for further 4.5 hours to incorporate ^{125}I-Urd into the cells. After the cultivation, the plate was washed with PBS (-), and was fixed with 10% TCA. The cells were dissolved in 1N NaOH and the amount of RI incorporated into the cells was measured using a γ-counter. The results are shown in Figure 1.

The relative activity at 1ng/ml and 3ng/ml was calculated with a two x two point method. The relative activity of the compound of Example 1 is 0.60, the relative activity of the compound of Example 2 is 0.42 and the relative activity of the compound of Example 3 is 0.50.

Example 5

Pharmocokinetics of the compound of Example 1 and 2 was studied. Wister rat (male, 11-week old) purchased from Nihon SLC was used.

1)Labeling HGF and the PEG-modified HGF

Each compound was labeled by the Iodogen method. The labeling was confirmed by way of treating the labeled compound with 10% trichloroacetic acid immediately after the labeling. As a result, more than 96% of isotope was recovered in the precipitate fraction. The labeled compound was frozen and stored at -80 °C.

2) Preparation of labeled compound for measurement of pharmacokinetics

A cold sample corresponding to the labeled compound was dissolved in PBS solution containing 2.5mg/ml HAS and 0.01 % Tween 80 to give a cold sample concentration of 0.25 mg/ml. A sample for pharmacokinetic test was prepared by adding the labeled compound obtained from trichloroacetic acid precipitate fraction (2.2×10^7 cpm) to the cold sample. The protein amount of labeled compound added to the sample for pharmacokinetic test was less than 4 % of the cold sample. Final concentration of HGF in the sample for pharamacokinetics test was 0.23mg/ml.

3) Administration to rats and preparation of blood sample

Each sample was administered to rats via a tail vein and an amount of administration was 2 ml/kg. The rats were divided to two groups (n = 2). After the administration, blood samples was collected from the orbital venous plexus at 2, 5, 10 and 30 minutes from one group and at 10, 20, 30, 45 and 60 from the other group.

4) Separation of serum and measurement of an isotope amount

The collected blood samples were separated to give serum. The serum (100 µl) was put into a tube, 10 % trichloroacetic acid was added and centrifuged, and the supernatant was removed to give a precipitate fraction. The amount of isotope in the precipitate was presumed to arise the labeled HGF and PEG-modified HGF, and measured by the γ-counter. From the results, the time-dependent concentration curves were calculated and are shown in Figure 2.

From the time-dependent curve, an initial concentration of the sample was derived and the amount of administration was confirmed.

The half life time of the each sample was calculated from Figure 2. The half life time of HGF was 59.2 minutes and

the half life times of the compound in Example 1 and 2 were 76.7 and 95.6 minutes, respectively. It was revealed that the PEG-modified HGF has a improved stability in vivo and prolonged activities.

Example 6

HGF modified by 3,4-bis-methoxypolyethyleneglycolhydrocinnamic acid

To a 10mM phosphate buffer solution (pH 6.5, 1M-NaCl, 0.01% Tween 80) (3.19ml) was added HGF (148.3mg) and 0.1 M borate buffer solution (11.64ml, pH8.21, 1M-NaCl) and the pH was adjusted to 8.21 with 1N NaOH solution. To the solution was added 3,4-bis-methoxypolyethyleneglycolhydrocinnamic acid N-hydroxysuccinimide ester (average molecular weight is about 10,000) (239.2mg, 0.3 equivalents to the amino groups) and the reaction mixture was stirred for 18 hours at room temperature. The pH of the reaction mixture was adjusted to 6.5 with 0.1 N HCl, and the reaction mixture was divided to 6 portions and each portion was purified by gel filtration chromatography (Column: Sephacryl S-200HR 2.6 cmØ x 90 cm, Eluent: 0.2M aqueous NaCl solution, Flow rate: 1.4ml/min., Detection wave length: 220nm). The objective fractions were collected and subjected to concentrating by ultrafiltration with YM-15 membrane (Amicon) to give an 0.15 M NaCl aqueous solution (21.0 ml) of titled compound (content of protein: 4.73 mg/ml, modifying rate of the amino groups; 29%).

Amino acid analysis

Asx 70.3 (83), Glx 55.9 (59), Ser 34.0 (38), Gly 56.0 (57), His 23.1 (22), Arg 40.7 (41), Thr 32.1 (38), Ala* 21.0 (21), Pro 38.2 (44), Tyr 27.7 (32), Val 26.8 (33), Met 18.1 (15), Ile 32.5 (37), Leu 33.9 (37), Phe 15.4 (17), Lys 42.5 (41), Cys - (40)

GPC

Column: TSK gel G3000SW 7.5 mmØ x 600 mm (manufacture by Toso Co., Ltd.)
Eluent: 10mM Tris buffer, 0.2M aqueous NaCl solution, 0.05%SDS
Flow rate: 0.6ml/min.
Detection wave length: 220 nm
Retention time: 17.95 min.

Example 7

To compare pharmaceutical activities of HGF and the PEG-modified HGF in Example 6, HGF and the PEG-modified HGF which have the same protein amount calculated as HGF were administered to rats and an enhancing activity on production of fibrinogen in liver was measured.

HGF and the PEG-modified HGF were dissolved in 10mM citric acid buffer solution (pH 5) containing 0.3 M NaCl and 0.01 % Tween 80, respectively. Administration doses of HGF and the PEG-modified HGF were 0.05, 0.15 and 0.5 mg of HGF protein/kg body weight. The sample was administered to rats via tail vein two times a day. One group consists of five rats. Next day of the two-day administration, rats were anesthetized and a blood sample was collected from abdominal vein. The sample was treated with citric acid and an amount of fibrinogen (mg/dl) was measured using automatic device for measuring blood coagulation (CA 5000, Cysmex).

The results are shown in Figure 3.

The activity of the samples was compared by the method of 2 x 2 parallel test at 0.3 mg/kg/day and 1 mg/kg/day. The PEG-modified HGF showed 8.6 times activity of HGF.

Claims

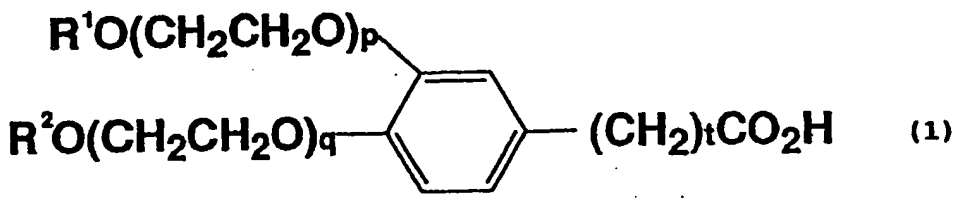
1. A PEG-modified HGF.

2. A PEG-modified HGF which comprises HGF modified by a PEG reagent that modifies an amino group of lysine and N-terminal of protein.

3. A PEG-modified HGF which comprises HGF modified by a PEG reagent that modifies an amino group of lysine and N-terminal of protein through an amide bond.

*: standard amino acid, (): calculated data, -: data not measured

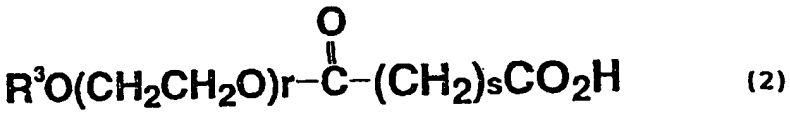
4. A PEG-modified HGF which is obtained by a process comprising a step of activating a carboxyl group of a PEG reagent represented by the formula (1):



(1)

(wherein R^1 and R^2 are same or different and independently a lower alkyl; p and q are are same or different and independently integer from 20 to 280; and t is 0 or a positive integer):
and a step of treating HGF with said activated reagent.

5. A PEG-modified HGF which is obtained by a process comprising a step of activating a carboxyl group of a PEG reagent represented by the formula (2):



(2)

(wherein R^3 is a lower alkyl, r is an integer from 20 to 280 and s is a positive integer); and
a step of treating HGF with said activated reagent.

6. A pharmaceutical composition for treating hepatic diseases, treating renal diseases, promoting growth epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cell, restoring hair, and in skin cosmetics, comprising an effective amount of the PEG-modified HGF as defined in claim 1, 2, 3, 4, or 5, and carrier, if necessary.

7. A method of using the PEG-modified HGF as defined in claim 1, 2, 3, 4 or 5 comprising manufacturing a pharmaceutical composition for treating hepatic diseases, treating renal diseases, promoting growth epithelial cells, treating cancer, reducing side effects of anti-cancer reagents, treating lung diseases, treating gastric and duodenal diseases, treating cerebral and nerval injury, increasing platelets, treating hypoptoteinemia, healing wounds, increasing hemopoietic stem cell, restoring hair, and in skin cosmetics.

Fig. 1

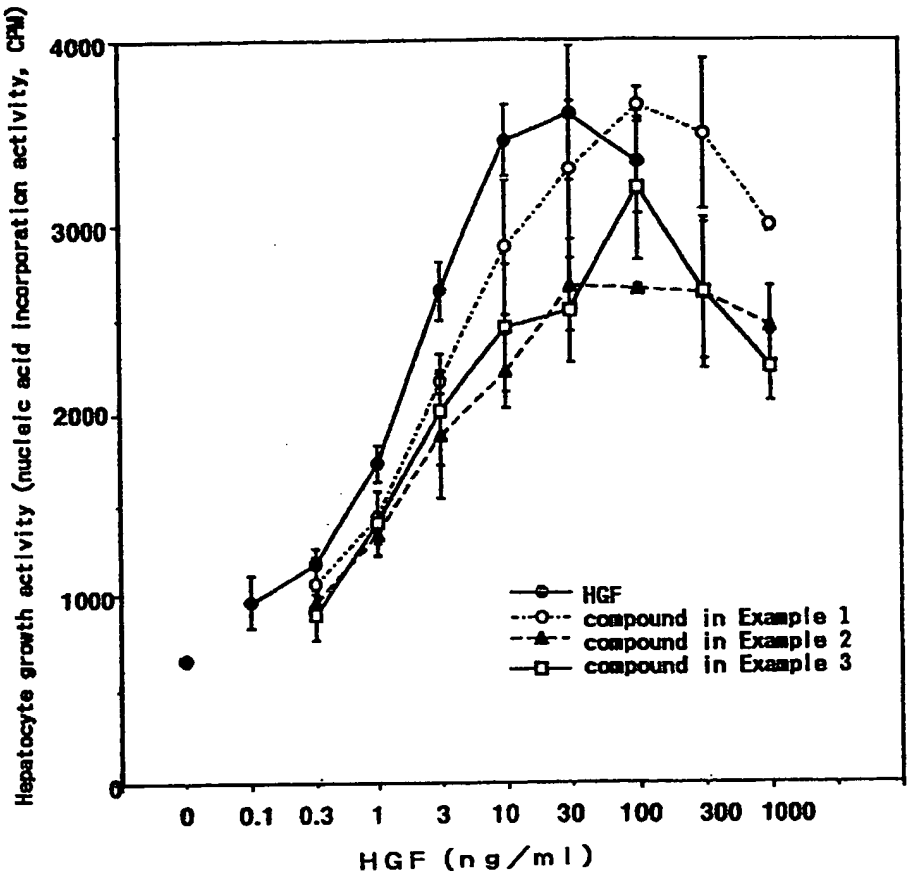

Hepatocyte growth activity (nucleic acid incorporation activity, CPM)
4000
3000
2000
1000
0
0
0.1
0.3
1
3
10
30
100
300
1000
HGF (ng/ml)
HGF
compound in Example 1
compound in Example 2
compound in Example 3

Fig. 2

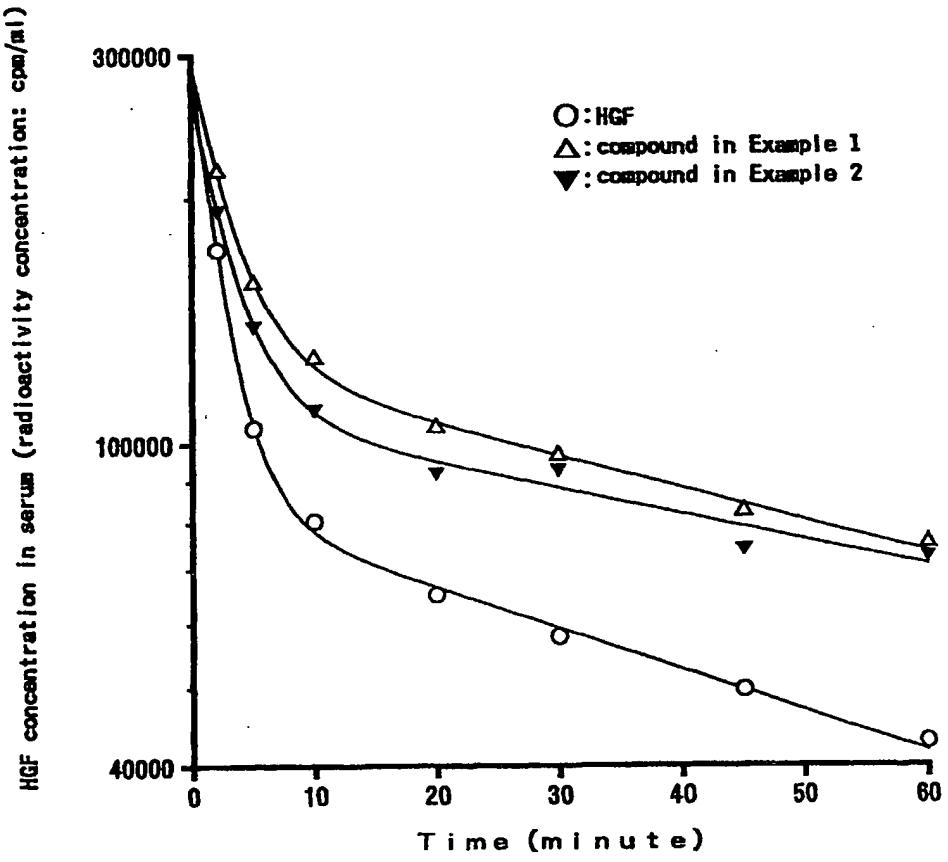
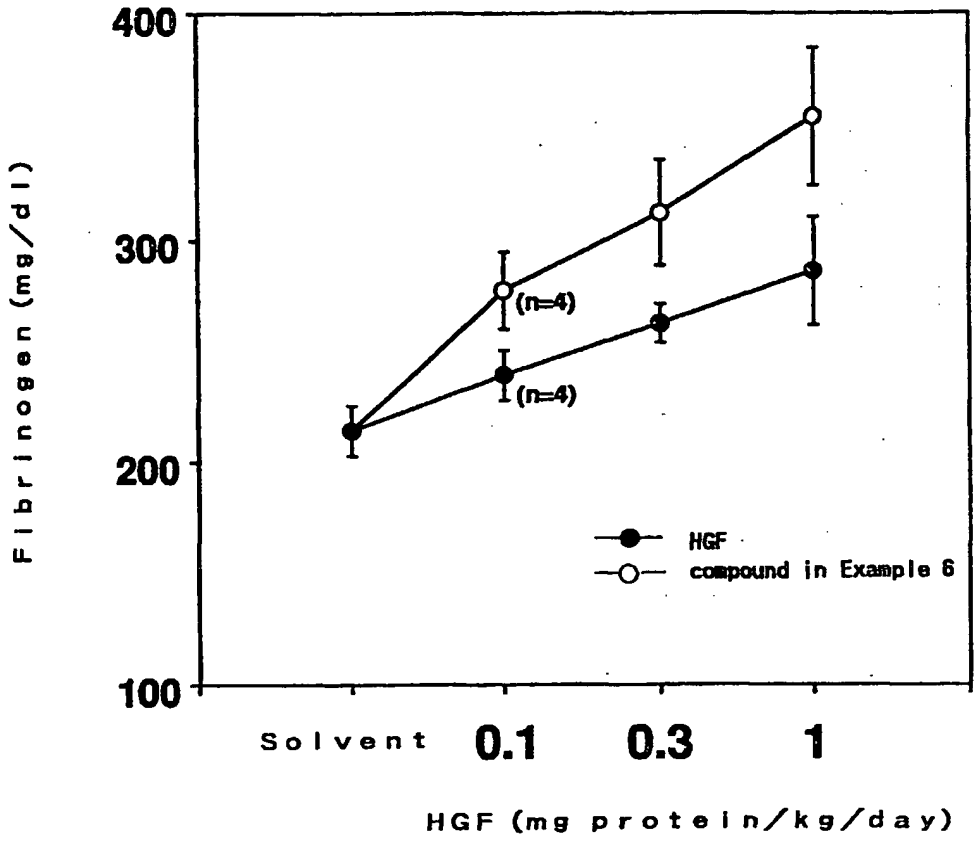

HGF concentration in serum (radioactivity concentration: cpm/ml)
300000
100000
40000
0
10
20
30
40
50
60
Time (minute)
○: HGF
△: compound in Example 1
▼: compound in Example 2

Fig. 3


400
300
200
100
Fibrinogen (mg/dl)
(n=4)
(n=4)
HGF
compound in Example 6
Solvent
0.1
0.3
1
HGF (mg protein/kg/day)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/00599

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^6 C07K17/08, C07K14/475, A61K38/18, A61K47/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int. Cl^6 C07K17/08, C07K14/475, A61K38/18, A61K47/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS ONLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO, 94/20069, A1 (AMGEN INC.), September 15, 1994 (15. 09. 94), Refer to claim, lines 5 to 16, page 9 & AU, 9463511, A1 & ZA, 9401571, A	1-3, 6-7 4 - 5
X Y	WO, 94/23740, A1 (CELTRIX PHARMACEUTICALS INC.), October 27, 1994 (27. 10. 94), Refer to claim, example & AU, 9465863, A1	1-3, 6-7 4 - 5
X Y	JP, 3-95200, A (Sumitomo Pharmaceuticals Co., Ltd.), April 19, 1991 (19. 04. 91), Refer to claim, examples 10, 13, 16, 42 to 44 (Family: none)	1-3, 6-7 4 - 5
Y	JP, 4-108827, A (Sumitomo Pharmaceuticals Co., Ltd.), April 9, 1992 (09. 04. 92), Refer to claim & EP, 473084, B1 & CA, 2050063, A & US, 5183660, A	4 - 5

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
June 4, 1996 (04. 06. 96)	June 11, 1996 (11. 06. 96)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/00599

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& DE, 69114614, A1	
Y	JP, 61-249388, A (Ajinomoto Co., Inc.), November 6, 1986 (06. 11. 86), Refer to claim, lines 10 to 16, lower right column, page 2 & EP, 200467, B1 & DE, 3676544, A1 & US, 5066590, A	4 - 5

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

Oncogene (1998) 17, 3045-3054

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Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor

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Invasion of various carcinoma cells follows their interaction with stromal cells. Hepatocyte growth factor (HGF), four-kringle-containing growth factor, is a mesenchymal or stromal-derived mediator which affects the growth and the invasiveness of carcinoma cells. We now have evidence that a four-kringle-containing antagonist for HGF, HGF/NK4 inhibits invasion of tumors *in vivo*, as well as *in vitro*. HGF/NK4 competitively inhibited the binding of HGF to Met/HGF receptors on GB-d1 human gallbladder carcinoma cells. HGF induced invasion of the cells through Matrigel basement membrane components and into collagen gels, but HGF-induced invasion was inhibited by HGF/NK4. Invasion of GB-d1 cells was induced by co-cultivation with stromal fibroblasts, which mimics tumor-stromal interaction, but it was almost completely suppressed by HGF/NK4. Likewise, invasive growth induced by HGF in collagen gels in GB-d1 cells, HuCC-T1 human cholangiocarcinoma cells, and ME-180 human uterus cervical carcinoma cells was also strongly inhibited by HGF/NK4. When GB-d1 cells were implanted subcutaneously into nude mouse, tumor cells invaded muscular tissue, but the infusion of HGF/NK4 inhibited this invasion. Furthermore, HGF/NK4 increased apoptotic cell death of GB-d1 cells and inhibited tumor growth *in vivo*. These results indicate that HGF/NK4 may inhibit growth and invasion of carcinoma cells, as mediated by HGF during tumor-stromal interactions. We propose that there is a unique therapeutic potential for HGF/NK4 to prevent tumor invasion and perhaps even metastasis.

Keywords: c-Met; hepatocyte growth factor; HGF-antagonist; tumor invasion; tumor-stromal interactions

Introduction

Most malignant tumors are carcinomas, and in most cancers, lethality is the result of local invasion and metastasis of neoplastic cells from the primary tumors to other tissues, during tumor progression. The invasive and metastatic characteristics of tumor cells depend on changes in the adhesive properties of tumor cells, degradation of the extracellular matrix (ECM), and a concomitant induction of cell movement (Hart *et al.*, 1989). Tumor growth also depends on angiogenesis, which again requires ECM degradation and migration of capillary endothelial cells (Folkman, 1989). Inhibition of a certain process in tumor invasion, metastasis, and angiogenesis may well lead to effective prevention or treatment of cancers.

Although changes in the proto-oncogene and tumor suppresser genes have been shown to lead to cellular transformation by a multistep process, the molecular mechanisms leading to tumor progression are still not clear. There is evidence that stromal cells influence the growth, invasion, and metastasis of tumor cells. The growth, migration, and invasion of tumor cells were markedly accelerated by a broad spectrum of fibroblasts *in vivo* (Camps *et al.*, 1990), as well as *in vitro* (Grey *et al.*, 1989). In addition to stromal involvement in tumor malignancy, several studies have shown that stromal alteration also occurs during tumor progression, including the existence of 'activated' fibroblasts surrounding tumor cells (van den Hoof, 1988; Grey *et al.*, 1989). Therefore, local and mutual interactions between carcinoma cells and stromal cells, i.e., tumor-stromal interactions, are of particular importance in regulating ECM degradation, invasion, and angiogenesis in tumor tissues.

Accumulating evidence has shown that hepatocyte growth factor (HGF) plays a distinct role in tumor-stromal interactions (Tajima *et al.*, 1992; Seslar *et al.*, 1993; Rosen *et al.*, 1994; Matsumoto *et al.*, 1996a; Nakamura *et al.*, 1997; Inoue *et al.*, 1997). HGF has potent motogenic activity (enhancement of cell movement) on a wide variety of cells, including various types of carcinoma cells, leading to the dissociation, scattering, and migration of cells (Weidner *et al.*, 1990; Jiang *et al.*, 1993a; Matsumoto *et al.*, 1994, 1996a; Jeffers *et al.*, 1996; Rosen *et al.*, 1996; Nakamura *et al.*, 1997; Inoue *et al.*, 1997). Fibroblast-derived factor which induces *in vitro* invasion of oral carcinoma was identified as HGF (Matsumoto *et al.*, 1994). Met/HGF receptor of membrane-spanning tyrosine kinase is expressed in a wide variety of tissues (mostly epithelial tissues) and carcinoma cells (Ebert *et al.*, 1994; Di Renzo *et al.*, 1995; Humphrey *et al.*, 1995; Natali *et al.*, 1996; Nagy *et al.*, 1996), while HGF is expressed in mesenchymal and stromal cells. Importantly, various types of carcinoma cells secrete factors which induce expression of HGF in fibroblasts (Seslar *et al.*, 1993; Rosen *et al.*, 1994; Matsumoto *et al.*, 1996a,b; Nakamura *et al.*, 1997). Therefore, the mutual interaction between carcinoma cells and stromal fibroblasts, mediated by carcinoma-derived HGF-inducers and stromal-derived HGF is likely to be a mechanism, leading to tumor progression. HGF, initially identified and cloned as a potent mitogen for

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Received 10 February 1998; revised 22 June 1998; accepted 22 June 1998

hepatocytes (Nakamura *et al.*, 1984, 1989; Miyazawa *et al.*, 1989), is a mesenchymal-(stromal-)derived multipotent factor which exhibits mitogenic, motogenic, and morphogenic activities. Functional coupling between HGF and Met/HGF receptor mediate epithelial-mesenchymal (or -stromal) interactions for the development and regeneration of organs and tissues (see reviews, Jiang *et al.*, 1993b; Zarnegar and Michalopoulos, 1995; Matsumoto and Nakamura, 1997). HGF also has angiogenic activity *in vitro* and *in vivo* (Bussolino *et al.*, 1992; Grant *et al.*, 1993).

Based on these notions, we considered that an antagonistic molecule which abrogates biological activities of HGF might have therapeutic effects to inhibit tumor growth, invasion and metastasis. We recently prepared an HGF-antagonist, designated 'HGF/NK4', which inhibits mitogenic, motogenic, and morphogenic activities of HGF (Date *et al.*, 1997). We now report that this HGF-antagonist (HGF/NK4) inhibits growth and invasion of tumor cells both *in vitro* and *in vivo*.

Results

Inhibition of HGF binding to the receptor and Met receptor tyrosine phosphorylation by HGF/NK4

In our recent report, we showed that the HGF-antagonist, HGF/NK4 inhibits mitogenic, motogenic, and morphogenic activities of HGF, using assay methods widely used to evaluate biological activities of HGF (Date *et al.*, 1997). In the initial experiments to know the effect of HGF/NK4 on tumor growth and invasion, we addressed whether antagonistic activity of HGF/NK4 would be also seen in tumor cells. To determine whether the HGF-antagonist, HGF/NK4 would competitively block the specific binding of HGF to the cell surface receptor, competitive binding analysis was carried out using ^{125}I-HGF in GB-d1 cells, a human gallbladder carcinoma cell line. Since the Kd value and the number of receptors for binding of HGF on GB-d1 cells were calculated to be 35.4 pM and 684 sites/cell, respectively (data not shown), the GB-d1 cells were incubated in the presence of 40 pM ^{125}I-HGF plus various concentrations of cold HGF or HGF/NK4. Addition of cold HGF competitively inhibited the specific binding of ^{125}I-HGF to the GB-d1 cells, and 50%-inhibition by cold HGF was seen with 40 pM HGF: the dose approximately equimolar to that of ^{125}I-HGF (Figure 1A). Addition of cold HGF/NK4 also inhibited the binding of ^{125}I-HGF to the cells. Inhibitory effects were seen from 10 pM HGF/NK4 and HGF/NK4 almost completely inhibited the ^{125}I-HGF binding at 40 nM; a 10^3-fold higher concentration than that of ^{125}I-HGF. The inhibition by 50% was seen with 400 pM HGF/NK4 and the concentration was 10-fold higher than that of HGF. Taken together, HGF/NK4 seems to competitively bind to the c-Met/HGF receptor with a 10-fold lower affinity than that of native HGF.

We then asked whether HGF/NK4 inhibits tyrosine autophosphorylation of Met induced by HGF. GB-d1 cells were serum-starved for 20 h and tyrosine phosphorylation of Met upon addition of HGF and/or HGF/NK4 was detected after immunoprecipitation

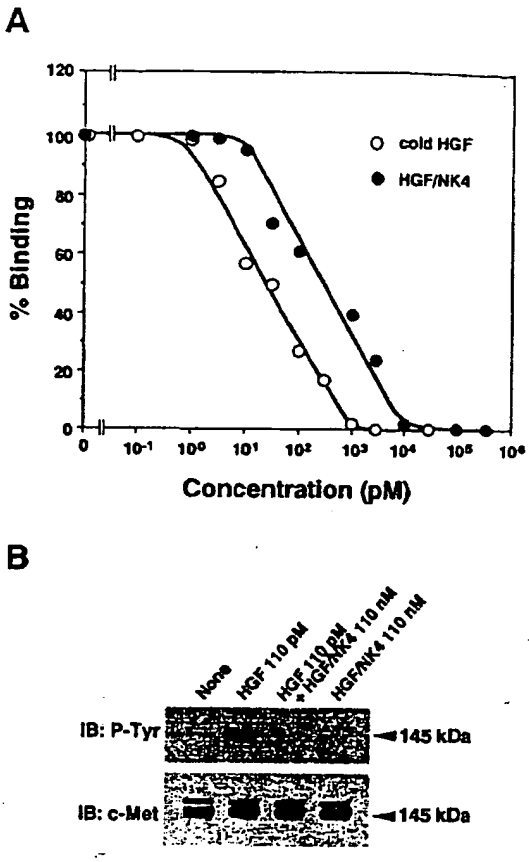

Figure 1 Replacement curve of ^{125}I-HGF binding to GB-d1 cells by unlabeled HGF or HGF/NK4 (A) and inhibitory effect of HGF/NK4 on tyrosine autophosphorylation of Met/HGF receptor (B). In (A), specific binding of 40 pM ^{125}I-HGF to GB-d1 cells was measured in the presence of various concentrations of unlabelled HGF or HGF/NK4. Each value represent the mean of quadruplicate experiments. In (B), HGF (110 pM) and/or HGF/NK4 (110 nM) were added to the cultures and cells were extracted 10 min later. The Met/HGF receptor was immunoprecipitated with anti-c-Met polyclonal antibody and electroblotted proteins were probed with anti-c-Met polyclonal antibody or anti-phosphotyrosine monoclonal antibody. IB, immunoblotting

and the following immunoblotting (Figure 1B). Tyrosine autophosphorylation of Met was detectable 10 min after adding 110 pM HGF, while simultaneous addition of HGF/NK4 and HGF inhibited tyrosine autophosphorylation (Figure 1B). HGF/NK4 alone did not induce tyrosine phosphorylation of Met/HGF receptor. Therefore, HGF/NK4 suppresses tyrosine phosphorylation of the c-Met/HGF receptor induced by HGF binding in GB-d1 cells.

HGF/NK4 inhibits invasion stimulated by HGF

Next, to examine whether HGF/NK4 inhibits tumor invasion, we measured the invasion of GB-d1 cells *in vitro* using a Matrigel invasion chamber and a collagen gel matrix. In the Matrigel invasion chamber, when the invasion of cells is enhanced, cells degrade the extracellular matrix components, and migrate through pores of membrane to the opposite side of the

membrane. In the absence of HGF, there was no evidence of invasive cells, however, HGF dose-dependently stimulated invasion of GB-d1 cells. Otherwise, HGF/NK4 itself did not stimulate the invasion, but it could block the invasion induced by HGF in a dose-dependent manner. The complete inhibition by HGF/NK4 was seen with a dose of 10-fold higher concentration of HGF (Figure 2). HGF/NK4 also inhibited cell scattering induced by HGF in GB-d1 cells (not shown). Likewise, with regards to growth promoting activity, 110 pM HGF enhanced cell proliferation by 1.34-fold, while the growth promoting activity of HGF was strongly inhibited by 110 nM HGF/NK4 (not shown).

To examine the invasion into the stroma, GB-d1 cells were plated on a collagen gel matrix. Even though GB-d1 cells were established from lymph node metastases and they invaded surrounding tissues when intradermally implanted into athymic nude mice, cells did not invade the collagen gel matrix. However, HGF markedly induced the invasion of GB-d1 cells into collagen gel matrix, when cells were initially plated on

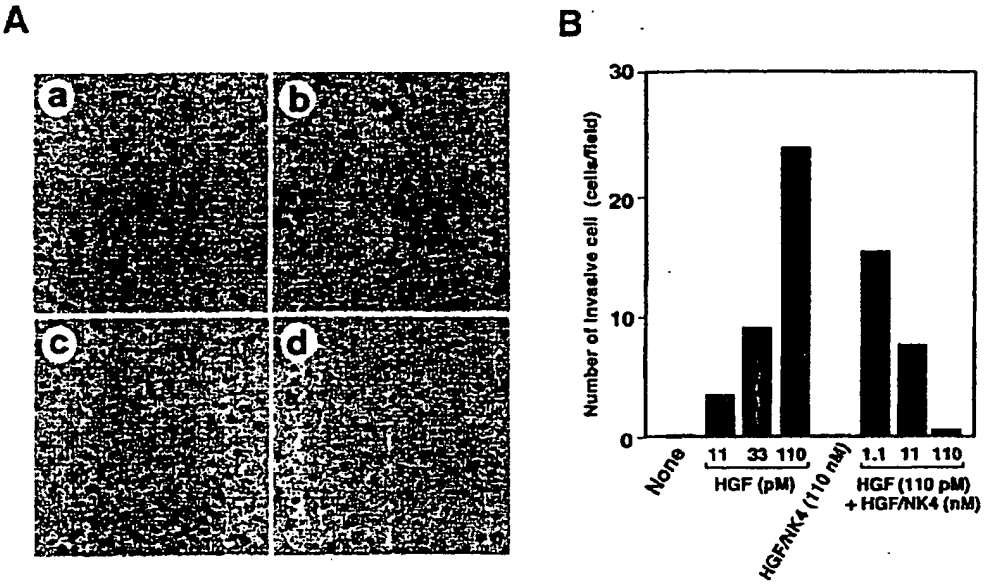

Figure 2 Inhibitory effect of HGF/NK4 on invasion of GB-d1 cells in Matrigel invasion chamber induced by HGF. (A) Appearance of GB-d1 cells invading the Matrigel to the lower side of a membrane with 8 μm pores. GB-d1 cells were cultured for 24 h in the absence (a) or presence of 110 pM HGF (b), 110 nM HGF/NK4 (c), or 110 pM HGF plus 110 nM HGF/NK4 (d). (B) The number of cells invading the Matrigel to the lower side of a membrane. Each value represents the mean of triplicate measurements

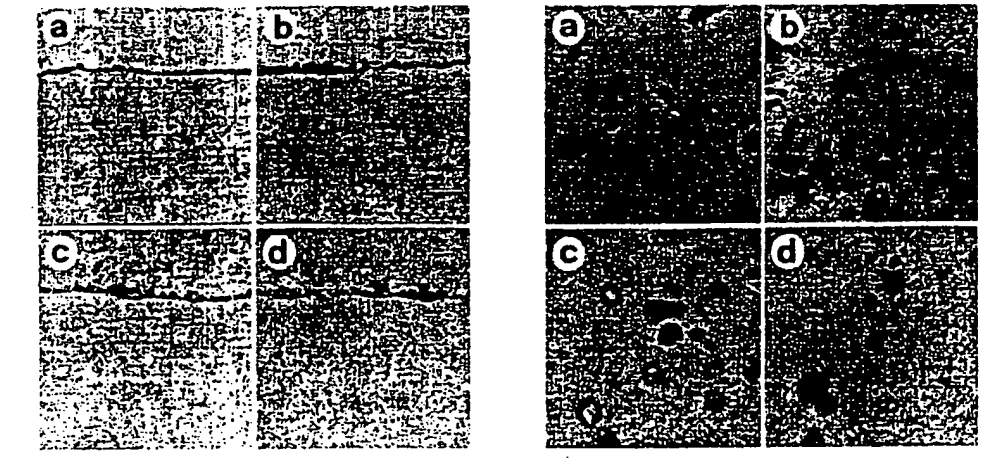

Figure 3 Inhibitory effect of HGF/NK4 on invasion of GB-d1 cells into the collagen gel, as induced by HGF. GB-d1 cells were grown on a collagen gel matrix (A) or in a collagen gel matrix (B) for 12 days in the absence (a) or presence of 110 pM HGF (b), 110 nM HGF/NK4 (c), or 110 pM HGF plus 110 nM HGF/NK4 (d)

collagen gels (Figure 3A). HGF/NK4 itself did not induce invasion into the collagen gel rather it almost completely blocked stromal invasion at a 10^3-fold higher concentration than HGF. Similarly, when GB-d1 cells were cultured in the collagen gels, the invasive growth induced by 110 pM HGF was almost completely inhibited by 110 nM HGF/NK4 (Figure 3B).

To determine if HGF also induces invasion of other carcinoma cells and to observe if HGF/NK4 would inhibit invasion of cells, HuCC-T1 human cholangiocarcinoma cells and ME-180 human uterus squamous carcinoma cells were cultured in collagen gels (Figure 4). In the absence of HGF, HuCC-T1 cells grew in collagen gels in a spherical multicellular structure, without invasion into gels (Figure 4). However, when cultured in the presence of HGF, HuCC-T1 cells invaded the gels multicellularly, which resulted in a branching duct formation in collagen gels. HGF/NK4 itself induced no change in appearance with a dose as high as 110 nM, whereas it almost completely inhibited invasion and branching duct formation of the cells induced by HGF, at a 10^3-fold higher concentration than HGF. Since HuCC-T1 cells are well-differentiated carcinoma cells originally derived from human hepatic bile duct, HGF seems to stimulate invasion of the cells, with concomitantly inducing morphogenic response in these cells. ME-180 cells grew in a spherical structure, without invasion into the gel (Figure 4), whereas in the presence of 110 pM HGF, the cells seemed to gain an invasive potential, which resulted in scattering of cell aggregates and the expression and invasion of cells into the gel. Addition of 110 nM HGF/NK4 alone did not induce such an invasive change, 110 nM HGF/NK4 almost completely inhibited invasive characteristics of ME-180 cells in the presence of 110 pM HGF. These observation indicate that HGF/NK4 inhibits *in vitro* invasion of distinct types of carcinoma cells.

To know whether the inhibitory effect of HGF/NK4 on invasion and growth of tumor cells might be related to a cytotoxicity or not, GB-d1, HuCC-T1, and ME180 cells were cultured in the absence or presence of HGF/NK4 for 24 h and the viability of cells was measured by trypan blue dye exclusion. HGF/NK4 did not exhibit significant change in viability of these cells (not shown), indicating that the inhibition of invasion and growth in these tumor cells by HGF/NK4 was not due to cytotoxic effect of HGF/NK4.

Inhibition of GB-d1 cell invasion through tumor-stromal interactions

We used a co-culture method in which fibroblasts derived from gallbladder stroma were cultured in the outer well, while GB-d1 cells were cultured in the Matrigel chamber (Figure 5A). We then measured invasion of GB-d1 cells as described above (Figure 5B and C). This co-culture method mimics the tumor-stromal interaction through basement membrane components.

When GB-d1 cells were cultured alone without fibroblasts, GB-d1 cells scarcely invaded, whereas HGF induced invasion of GB-d1 cells. In contrast, GB-d1 cells invaded when they were cultured with fibroblasts. The number of invaded cells was higher than that seen in the presence of 10 ng/ml HGF. Since HGF concentration in the co-culture system was 8 ng/ml, as measured by enzyme-linked immunosorbent assay, the finding suggests that fibroblast might secrete a factor(s) distinct from HGF and such a factor(s) stimulates invasion of the cells in this system. Importantly, HGF/NK4 dose-dependently inhibited the invasion and almost completely inhibited the invasion of the cells at 110 nM (Figure 5B and C). Since HGF/NK4 almost completely blocked invasion of the GB-d1 cells in co-culture condition, the stimulatory effect of a fibroblast-derived factor(s) is likely to be dependent on the co-existence of HGF, in stimulating invasion of GB-d1 cells. Although we have yet to specify such a fibroblast-derived factor(s), one possible explanation is that fibroblasts might produce a cell motility factor, yet such a factor alone could not induce tumor invasion through basement membrane. In the co-existence of HGF, however, HGF up-regulates proteinases responsible for tumor invasion through ECM components, and in this condition, fibroblast-derived motility factor might stimulate

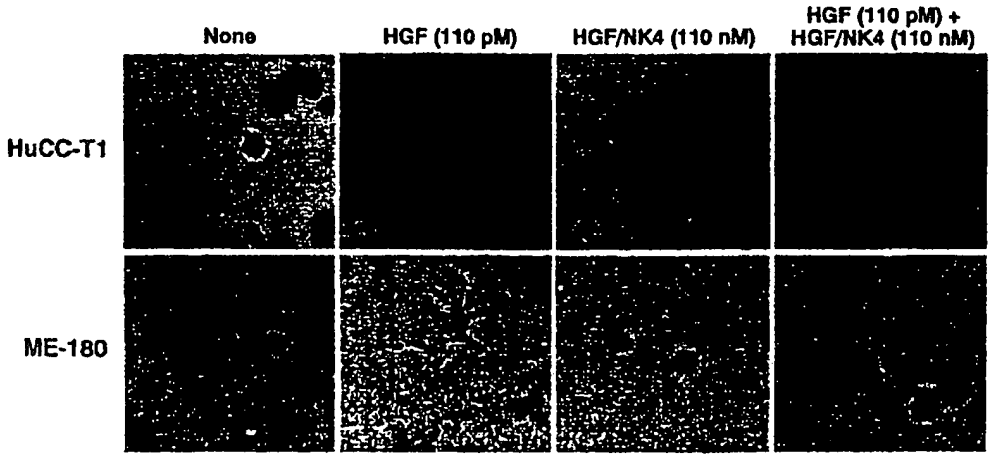

Figure 4 Inhibitory effect of HGF/NK4 on invasion of HuCC-T1 and ME-180 cells in collagen gels, as induced by HGF. HuCC-T1 and ME-180 cells were cultured in a collagen gel matrix for 12 days in the absence or presence of 110 pM HGF, 110 nM HGF/NK4, or 110 pM HGF plus 110 nM HGF/NK4

tumor invasion in an additive manner with HGF. Another possibility is that fibroblasts might produce pro-matrix metalloproteinases (pro-MMPs), but the activation of pro-MMPs might depend on tumor-derived proteinase such as uPA. Indeed, HGF strongly stimulates uPA activity in GB-d1 cells.

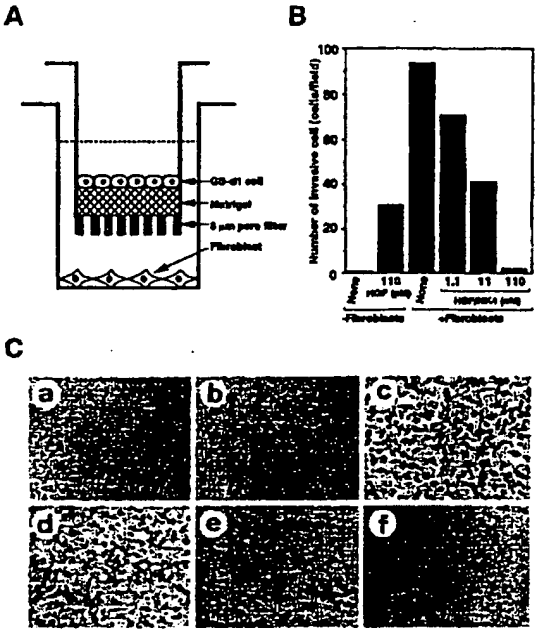

Figure 5 Inhibition of *in vitro* invasion of GB-d1 cells co-cultured with stromal fibroblast. (A) Diagram for co-culture method of GB-d1 cells and gallbladder fibroblasts. (B) The number of cells invaded cells. Each value represents the mean of triplicate experiments. (C) Appearance of invaded GB-d1 cells. GB-d1 cells were cultured for 24 h in absence (a) or presence of 110 pM HGF, without fibroblasts (b), or they were co-cultured with fibroblasts in the absence (c) or presence of 1.1 nM HGF/NK4 (d), 11 nM HGF/NK4 (e), or 110 nM HGF/NK4 (f)

Inhibition of MMP-9 and urokinase-type plasminogen activator by HGF/NK4

Since gelatinases are involved in tumor invasion, particularly through basement membrane components, we measured gelatinase activity, using GB-d1 cells. Cells were incubated with HGF and conditioned medium was subjected to the zymography. In control culture without HGF, cells secreted MMP-9 (92 kDa gelatinase) (Figure 6A), but we could scarcely detect other gelatinases in this condition (not shown). Activity of MMP-9 was dose-dependently stimulated by HGF, however, HGF/NK4, as well as anti-HGF antibody, inhibited the stimulatory effect of HGF on MMP-9 activity.

Since the possible role of uPA in tumor invasion and activation of pro-MMPs is well recognized (Vassalli *et al*., 1994), we also measured uPA activity in conditioned media from GB-d1 cells (Figure 6B). As shown in Figure 6B, HGF but not HGF/NK4 strongly stimulated uPA activity, whereas HGF/NK4 almost completely inhibited uPA activity, induced by HGF mostly to the basal level.

In vivo *inhibition of tumor growth and invasion*

Based on *in vitro* inhibition of tumor invasion by HGF/NK4, we next examined whether HGF/NK4 would inhibit tumor invasion and growth *in vivo* (Figure 7). GB-d1 cells were subcutaneously implanted into nude mice and HGF/NK4 or vehicle alone was continuously infused for 2 weeks into a subcutaneous region near the tumor mass, one week after the implantation. Tumor volume increased rapidly following one week after implantation, however, the increase in tumor volume was strongly inhibited by the infusion of HGF/NK4 (Figure 7A). In control tumor tissue infused with vehicle alone, GB-d1 cells invaded surrounding tissues, particularly into muscle tissue (Figure 7B, a and c). In contrast, invasion of the tumor cells was not seen in mice infused with HGF/NK4 (Figure 7B, b and d). The similar histological

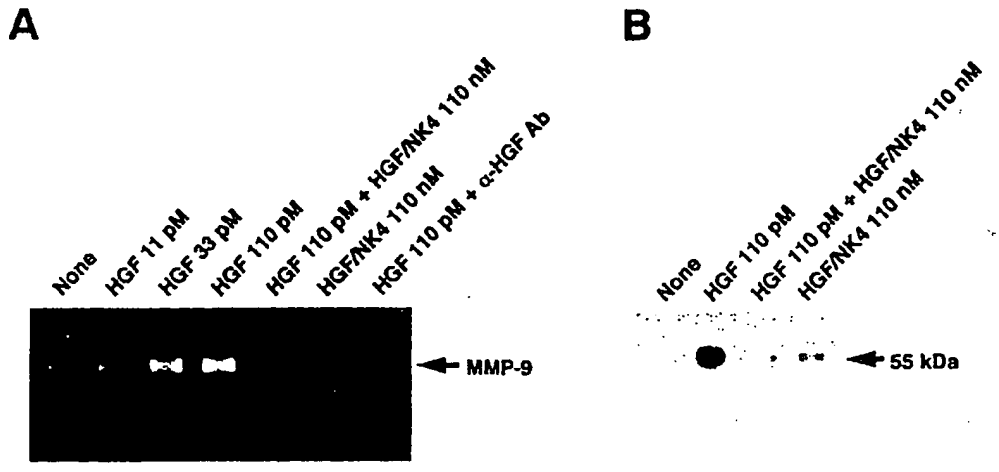

Figure 6 Inhibition of MMP-9 and uPA activities in GB-d1. MMP-9 activity (A) and uPA activity (B) were measured in conditioned media from GB-d1 gallbladder cancer cells. GB-d1 cells were cultured for 24 h in the absence, or presence of HGF and/or HGF/NK4, and conditioned media were subjected to SDS-PAGE. Anti-HGF antibody (α-HGF Ab) was added to the culture at 10 μg/ml in the presence of 110 pM HGF in (A)

observation that the infusion of HGF/NK4 inhibited invasive growth of GB-d1 cells into surrounding tissues was seen in tumor tissues in other mice (not shown).

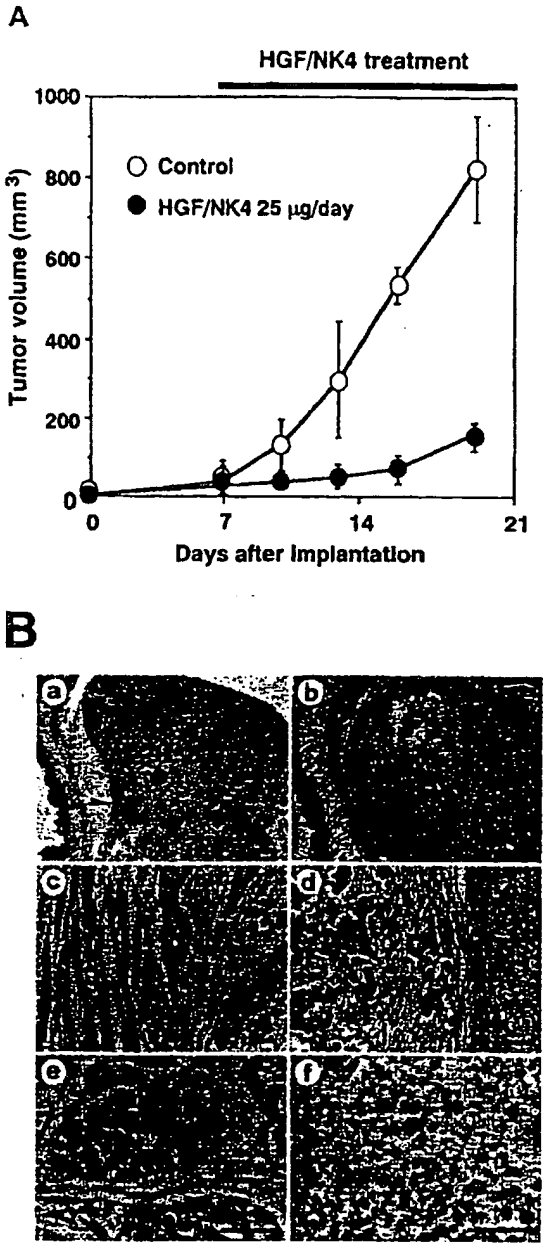

Figure 7 Inhibition of growth and invasion of GB-d1 cells in nude mice by infusion of HGF/NK4. Increase in tumor volume (A) and histology of tumor tissues (B) were shown. One week after implantation of GB-d1 cells (5×10^6) into nude mice, HGF/NK4 solution or vehicle alone was infused for two weeks into subcutaneous regions near the tumor mass using osmotic pump. Twenty-one days after implantation of the tumor cells, tumors were resected and examined histologically. Five mice were used in each experimental group and values are expressed as mean ± s.d. ($n = 5$). In (B), typical appearance of tumor tissues (T) in a control mouse not given HGF/NK4-infusion were shown in a, c, and e, while the appearance in mice given HGF/NK4-infusion were shown in b, d, and f, respectively. Evidence of muscle invasion (arrow) is shown in a and c. Bars represent 500 μm in a and b, and 50 μm in c, d, e, and f, respectively

Tumor growth is generally regulated by a counterbalance between cell proliferation and cell death. To address a mechanism by which HGF suppressed tumor growth, apoptotic cells and proliferating cells were respectively detected by TUNEL method and immunohistochemical staining for PCNA (Figure 8). In control tumor tissues, 4.0% of tumor cells underwent apoptotic cells death (Figure 8A). When HGF/NK4 was infused into mice, the number of apoptotic cells increased to 11.0%, a value being 2.7-fold higher than that in control tumor tissues. On the other hand, the number of PCNA-positive cells in tumor tissues infused with HGF/NK4 was decreased to 76% of the value in control tumor tissues. The weak inhibitory effect of HGF/NK4 on proliferation of the tumor cells seems to be consistent with *in vitro* finding that HGF enhanced cell proliferation by 1.34-fold, while the weak mitogenic activity of HGF was inhibited by HGF/NK4 (not shown). Consistent with the increase in TUNEL-positive apoptotic tumor cells in mice infused with HGF/NK4, histological appearance of tumor tissues indicate that a significant population of tumor cells underwent apoptotic cell death in HGF/NK4-

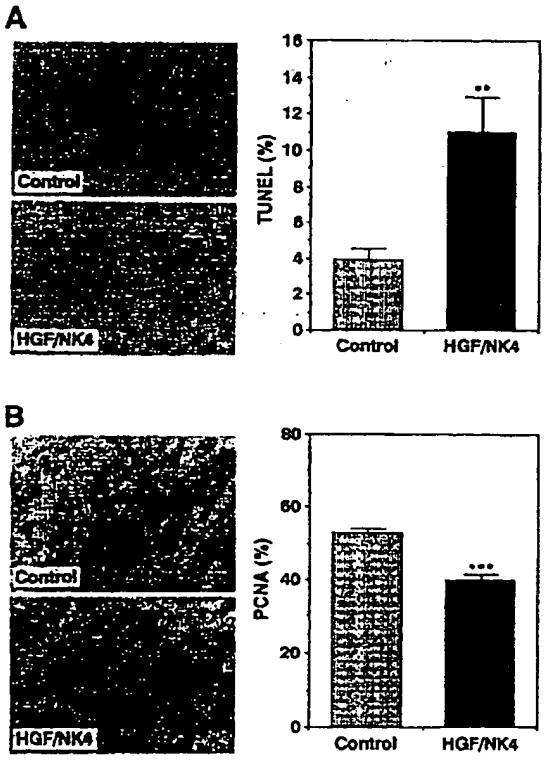

Figure 8 Changes in apoptotic and proliferating cells in GB-d1 gallbladder carcinoma cells in nude mice by infusion of HGF/NK4. Distribution and changes in the number of apoptotic cells (A) and proliferating cells (B) were shown. One week after implantation of GB-d1 cells (5×10^6) into nude mice, HGF/NK4 solution (HGF/NK4) or vehicle alone (control) was infused for two weeks into subcutaneous subcutaneous regions near the tumor mass using osmotic pump. Twenty-one days after the implantation, tumors were resected and subjected to histological analysis. Apoptotic cells and proliferating cells (arrowheads) were respectively detected by TUNEL method and PCNA staining. Five mice were used in each experimental group and values are expressed as mean ± s.d. (**, $P < 0.01$; ***, $P < 0.001$)

treated mice (Figure 7B, b and f): there were numerous picnotic cells, a typical histological feature of apoptosis, in the central region of the tumor mass. There was no such a massive apoptosis in tumor tissues in control mice (Figure 7B, a and e).

Discussion

During malignant progression, tumor cells acquire characteristics that promote metastasis. In the case of carcinomas, these properties include autonomous cell proliferation, invasion through basement membrane and stromal ECM, enhanced cell motility, entering into circulatory system, arrest in target organs, and the formation of a secondary outgrowth. With insight into molecular mechanisms of invasion and metastasis, one can devise ways to block these processes, a prospect that has therapeutic potential for treatment of malignant tumors. In the present study, we demonstrated that a specific antagonist for HGF, HGF/NK4 potently inhibited not only *in vitro* invasion of tumor cells, as induced through co-cultivation with fibroblasts, as well as HGF, but also *in vivo* growth and tumor invasion into surrounding host tissues.

HGF is proteolytically processed to a heterodimer molecule composed of a 69 kDa α-chain and 34 kDa β-chain from a single-chain precursor, and the α-chain contains the N-terminal hairpin domain and subsequent four kringle domains (Nakamura *et al.*, 1989). Previous studies revealed that the N-terminal hairpin domain, the first kringle (K1), and second kringle (K2) are responsible for high-affinity binding of HGF to Met/HGF receptor (Hartmann *et al.*, 1992; Lokker *et al.*, 1992, 1993; Okigaki *et al.*, 1992; Mizuno *et al.*, 1994). Small molecules consist of the N-terminal hairpin domain and K1 or K1K2, designated HGF/NK1 and HGF/NK2 exist as naturally synthesized variants (Chan *et al.*, 1991; Miyazawa *et al.*, 1991; Cioce *et al.*, 1996), and these variants have partial agonistic activities (Hartmann *et al.*, 1992; Cioce *et al.*, 1996). Likewise, HGF/NK3 has no mitogenic activity on endothelial cells, while it stimulates migration of the cells (Silvagno *et al.*, 1995). Schwall *et al.*, (1995) also reported that heparin induces dimerization of HGF/NK1 and HGF/NK2 and confer their mitogenic activity. In contrast, we recently found that HGF/NK4 composed of the N-terminal hairpin domain and four kringle domains has no agonistic activities, in terms of mitogenic, motogenic and morphogenic activities, and the tyrosine phosphorylation of Met/HGF receptor (Date *et al.*, 1997). HGF/NK4 binds to the Met/HGF receptor, but competitively inhibits the multiple biological activities of HGF. Moreover, HGF/NK4 has no biological activities even in the presence of heparin (not shown). While the role of K4 in the antagonistic activity of HGF/NK4 remains to be addressed, HGF/NK4 seems to be the first available complete antagonist for the multiple activities of HGF.

Regulation of cell growth, cell movement, morphogenesis, and accompanying remodeling of ECM is a crucial feature for construction of tissue structures. HGF is a mesenchymal- or stromal-derived mediator which regulates cell growth, cell motility and morphogenesis during tissue formation and repair (see reviews, Zarnegar and Michalopoulos, 1995; Matsumoto and Nakamura, 1997). Given that various types of carcinoma cells utilize reminiscent mechanisms in epithelial-mesenchymal interactions, HGF might play a role in tumor-stromal interaction, and studies elucidated several mechanisms for the involvement of HGF in tumor progression through tumor-stromal interaction (Matsumoto *et al.*, 1994, 1996a,b; Inoue *et al.*, 1997; Nakamura *et al.*, 1997; Yamamoto *et al.*, 1997). Various types of carcinoma cells produce inducers for HGF in fibroblasts (Seslar *et al.*, 1993; Rosen *et al.*, 1994; Matsumoto *et al.*, 1996a,b; Nakamura *et al.*, 1997), and enhanced invasive properties by HGF were demonstrated in a wide variety of carcinoma cells (Weidner *et al.*, 1990; Jiang *et al.*, 1993b; Matsumoto *et al.*, 1994, 1996a; Jeffers *et al.*, 1996; Rosen *et al.*, 1996; Nakamura *et al.*, 1997; Inoue *et al.*, 1997). Autocrine activation of the Met/HGF receptor in leiomyosarcoma cells induced enhanced tumorigenicity, invasion and metastasis (Jeffers *et al.*, 1996). If a paracrine or autocrine mode of HGF activation in tumor tissues could be blocked, then the invasive growth of tumors could be inhibited. Our present study demonstrated that the HGF/NK4 inhibits invasive growth of tumor cells both *in vitro* and *in vivo*.

HGF activates multiple pathways in target cells, leading to decreased cell-cell adhesions and enhanced cell motility and invasiveness. HGF induces tyrosine phosphorylation of β-catenin (Shibamoto *et al.*, 1994) and focal adhesion kinase ($p125^{FAK}$) (Matsumoto *et al.*, 1994), and activates rho small GTP-binding protein (Takaishi *et al.*, 1994; Ridly *et al.*, 1995), events that lead to increased cell motility and dissociation of cell-cell interactions. In addition to increased cell motility, activation and/or induction of specific proteases which mediate the degradation of ECM components promote tumor invasion. In this context, HGF induces uPA-related proteolysis network in certain types of cells (Pepper *et al.*, 1992), and Jeffers *et al.*, (1996) demonstrated that induction of the uPA proteolysis network by HGF is coupled to enhanced tumorigenecity and invasive and metastatic properties in certain tumor cells. We here found that HGF stimulates MMP-9 and uPA activities in gallbladder cancer and that the induction was blocked by HGF/NK4. It is highly probable that HGF/NK4 inhibits HGF-triggered multiple events that lead to enhanced motility and degradation of basement membrane and ECM components both *in vitro* and *in vivo*.

It is noteworthy that infusion of HGF/NK4 suppressed the increase in tumor mass and that HGF/NK4 increased apoptotic tumor cells while weakly decreased proliferating tumor cells. The inhibitory effect of HGF/NK4 on *in vivo* tumor growth is thus likely to be achieved by both increased apoptosis and decreased proliferation of tumor cells, wherein the increased apoptosis by HGF/NK4 may be more predominant cause for tumor suppression than the decreased cell proliferation. *In vivo* growth of tumor cells particularly depends on their ability to induce angiogenesis in tumor tissues (Folkman, 1989) and inhibition of tumor angiogenesis results in tumor suppression through inducing apoptosis of tumor cells, rather than inhibiting tumor cell proliferation (O'Reilly *et al.*, 1996). HGF is known as an angiogenic factor (Bussolino *et al.*, 1992; Grant *et al.*, 1993) and breast carcinoma cells capable of producing HGF induced

more extensive angiogenesis *in vivo* than that seen in parental cells incapable of producing HGF (Lamszus *et al*., 1997). Taken together, we speculate that inhibition of angiogenic activity, as well as mitogenic activity of HGF may be the cause for suppression of *in vivo* tumor growth by HGF/NK4.

Inhibition of tumor invasion and metastasis as well as tumor angiogenesis awaits the development of new strategies in cancer treatment and prevention. Based on the notion that HGF is a mediator in tumor-stromal interactions which confer malignant characteristics in tumor cells, we demonstrated for the first time that the antagonistic molecule for HGF, HGF/NK4 practically inhibits *in vivo* growth and invasion of a certain type of tumor cells, in experimental animals. Together with findings that the coupling of HGF and Met/HGF receptor affect invasive properties in a wide variety of tumor cells, application of the HGF-antagonist or possibly its gene may provide a new strategy toward cancer prevention.

Materials and methods

Materials

Human recombinant HGF was purified from the conditioned medium of Chinese hamster ovary cells transfected with human HGF cDNA (Nakamura *et al*., 1989; Seki *et al*., 1990). Polyclonal antibody against human HGF was prepared from the serum of a rabbit immunized with human recombinant HGF. IgG was purified using protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) and anti-human HGF IgG (1 μg/ml) completely neutralized the biological activities of 1 ng/ml human HGF. HGF/NK4 was prepared by proteolytic digestion with elastase as described elsewhere (Date *et al*., 1997). Antiphosphotyrosine monoclonal antibody (PY-20) was obtained from Chemicon International, Inc. (Temecula, CA) and anti-c-Met polyclonal antibody (C-12) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture

GB-d1 cells were established from surgical specimens from a 58-year-old man with gallbladder cancer (Shimura *et al*., 1995). Gallbladder fibroblasts were initially proliferated outward from surgical specimens from a 66-year-old woman with cholelithiasis (unpublished data), and used during at 10–15 passages. ME-180 human cervical epidermoid carcinoma cells and HuCC-T1 human cholangiocellular carcinoma cells were obtained from the Japanese Cancer Research Resources Bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin, penicillin and 10% fetal calf serum (FCS).

Receptor assay

HGF was radioiodinated by the chloramine-T method, as described elsewhere (Nakamura *et al*., 1997). The specific activity of ^{125}I-HGF was 30–54 μCi/μg protein. GB-d1 cells were cultured on a 24-well plate dish, the cultures were washed once with binding buffer (Hanks' solution containing 20 mM HEPES and 2 mg/ml bovine serum albumin, pH 7.0) and equilibrated in the same buffer for 30 min at 12°C. Ice-cold binding buffer containing 40 pM ^{125}I-HGF, with increasing concentrations of unlabeled HGF or HGF/NK4, was added and the preparation was incubated for 1 h. Cultures were washed three times with ice-cold binding buffer, and the ^{125}I-HGF specifically bound to the cells was measured using a γ-counter after lysis of the cells with 1 M NaCl. All binding experiments were performed in quadruplicate.

Measurement of in vitro *tumor invasion*

In vitro invasion of tumor cells was measured using a Matrigel invasion chamber (Becton Dickinson, Bedford, MA) or collagen gel. When using a Matrigel chamber, GB-d1 cells suspended in DMEM containing 10% FCS were added to inner cup of a Matrigel invasion chamber at a density of 1×10^4 cells/cm^2 and HGF and/or HGF/NK4 were added to the medium of the outer cup. After 24 h cultivation, cells which migrated through Matrigel and filter membrane with 8-μm pores were counted after staining with hematoxylin. For co-cultivation of GB-d1 cells and gallbladder fibroblasts, human gallbladder fibroblasts were initially seeded on 24-well plates (Costar) at a density of 10^5 cells/cm^2 and cultured in DMEM containing 10% FCS for 24 h. The medium was changed to fresh medium supplemented with 2% FCS, GB-d1 cells were seeded on the inner cup of 24-well Matrigel invasion chamber at a density of 5×10^4 cells/cm^2 and cultured for 24 h.

For invasion assay in a collagen gel, seven volumes of type I collagen solution (Cellmatrix, type IA, Nitta gelatin, Yao, Japan) were mixed with two volumes of fivefold concentrated DMEM and one volume of FCS, under ice-cooling, added to an inner cup of a Transwell chamber (Costar), and incubated for 10 min at 37°C. GB-d1 cells plated on the top of a 1 mm thick collagen gel at a density of 10^5 cells/cm^2 were cultured for 8 h. The medium was replaced with DMEM supplemented with 10% FCS and the cells were cultured in the presence of HGF and/or HGF/NK4 in the medium for 12 days. After fixing in 70% ethanol, the cells were embedded in paraffin. Sections were stained with hematoxylin and eosin. Alternatively, GB-d1, ME-180, and HuCC-T1 cells were harvested using trypsin-EDTA solution, suspended in ice-cold 0.2% collagen solution at a density of 10^6 cells/ml, and 500 μl aliquots of cell suspension were added to wells with a 16 mm width (Nunc, Kampstrup, Roskilde, Denmark). After the collagen solution gelled, 500 μl of culture medium containing HGF and/or HGF/NK4 was added to each well. Culture medium was changed every 3 days, and the cells were cultured for 12 days.

Detection of receptor tyrosine autophosphorylation

Tyrosine phosphorylation of Met/HGF receptor was analyzed as described elsewhere (Date *et al*., 1997). Briefly, serum-starved cells were treated with HGF and/or HGF/NK4, and washed with phosphate-buffered saline (PBS) containing 1 mM Na_3VO_4. The cells were extracted in lysis buffer composed of 20 mM Tris-HCl (pH 7.4), 10 mM EDTA, 150 mM NaCl, 2 mM Na_3VO_4, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100, and the lysate was centrifuged at 12 000 *g* for 10 min. The supernatant was treated with antibody to human c-Met and Protein A-Sepharose and the immunoprecipitates were separated by SDS–PAGE. The proteins were electroblotted onto PVDF membranes (Bio-Rad, Hercules, CA, USA), and probed with anti-phosphotyrosine antibody or anti-c-Met antibody. Proteins reacting with these antibodies were detected using an ECL enhanced chemiluminescence method (Amersham, Little Chalfont, England).

Zymographies for gelatinase and uPA activities

Subconfluent GB-d1 cells were washed three times with PBS, and were cultured in serum-free DMEM, with or

without HGF and/or HGF/NK4 for 24 h. The conditioned medium was collected and concentrated 10-fold with Centricon-10 concentrators (Amicon, Beverly, MA, USA). For the measurement of gelatinase activity, samples were mixed with SDS sample buffer, and subjected to SDS-PAGE, using 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gel was washed in 2.5% Triton X-100, and incubated in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM $CaCl_2$ and 1 mM $ZnCl_2$ for 20 h at 37°C. The gel was stained with 1% Coomassie brilliant blue and photographed. For the measurement of uPA activity, cell extracts and culture supernatants were prepared and analyzed by zymography, as described (Vassalli *et al.*, 1984).

In vivo *assay for tumor invasion and histochemical analysis.*

Six to 8-week-old male nude mice (BALB/c nu/nu, Japan SLC, Inc., Hamamatsu, Japan) were subcutaneously implanted with 5×10^6 GB-d1 cells. After one week, an osmotic pump (Alzet 2002, Palo Alto, CA) containing HGF/NK4 or vehicle alone (10 mM phosphate buffer, pH 7.3, containing 1 M NaCl, and 0.01% Tween 80) was surgically implanted near the tumor and HGF/NK4 solution or vehicle was continuously infused for two weeks into the subcutaneous region near the tumor mass. Five mice were used in each experimental group. The size of tumors in all groups was measured using a dialcaliper, and the volume of tumors was determined using the formula width^2 × length × 0.5. All mice were killed 14 days after osmotic pump implantation (21 days after tumor implantation), the tumors were excised and weighted, then were fixed in 10% formalin for 1 day and embedded in paraffin according to standard histological procedures. Sections were stained with hematoxylin and eosin.

To detect apoptotic cells in tumor tissues, tissue sections were stained by a modified TUNEL method, using ApopTag *in situ* Apoptosis Detection System (Oncor, Gaithersburg, MD). Proliferating cells were detected using antibody against proliferating cell nuclear antigen (PCNA). Tissue sections were treated with anti-PCNA antibody (PC-10, Novocastra Lab., Newcastle), followed by peroxidase-conjugated avidin-biotin complex, using Vectastain Elite ABC kit (Vector, Burlingame, CA, USA). The number of TUNEL-positive apoptotic cells and PCNA-positive proliferating cells were respectively determined by counting more than 1000 nuclei in different sections in each animal.

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Site-Specific Attachment of Functionalized Poly(ethylene glycol) to the Amino Terminus of Proteins

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A convenient method for the construction of site-specifically modified poly(ethylene glycol)–protein conjugates is described. This method relies on the ability to generate a reactive carbonyl group in place of the terminal amino group. If the protein has N-terminal serine or threonine, this can be done by very mild periodate oxidation and generates a glyoxylyl group. A method less restricted by the nature of the N-terminal residue, but which requires somewhat harsher conditions, is metal-catalyzed transamination, which gives a keto group. The N-terminal-introduced reactive carbonyl group specifically reacts, under mild acidic conditions, with an aminooxy-functionalized poly(ethylene glycol) to form a stable oxime bond. Using polymers of different size and shape (linear or multibranched), various conjugates of IL-8, G-CSF, and IL-1ra were constructed and further characterized with respect to their biological activity and pharmacokinetic behavior in rats. Unlike most previous methods, this approach places a single PEG chain at a defined site on the protein. It should therefore be more likely to conserve biological activity when the latter depends on interaction with another macromolecule (unlike enzymic activity which often survives multiple PEGylation).

INTRODUCTION

Covalent attachment of monomethoxypoly(ethylene glycol) to therapeutic proteins prolongs their circulatory life time *in vivo*, reduces their antigenicity and immunogenicity, and improves their resistance to proteolysis (Davis et al., 1980; Abuchowski et al., 1984; Katre et al., 1987). These properties are of great clinical interest, especially in the case of relatively small proteins, where it is believed that an increase of the Stoke's radius is consistent with a reduced renal clearance. PEG functionalization methods have been the subject of a recent review (Zalipsky, 1995). When applied in protein modification, a reactive functional group such as trichloro-*s*-triazine (Abuchowski et al., 1977), carbonylimidazole (Beauchamp et al., 1983), and succinimidyl succinate (Abuchowski et al., 1984) is introduced to link the polymer to the ϵ-amino group of lysine residues. All involve the preparation of an activated PEG with a reactive functional group which can be coupled to the lysine groups on proteins. Problems in controlling the conjugation chemistry frequently arise because the number and location of lysine residues vary greatly in native proteins and because the location of reactive groups on protein is random. Thus, the stoichiometry of the protein–polymer conjugate and the attachment sites of the polymer to the protein cannot be precisely controlled, factors which might have important implications for protein stability and function. These limitations can be circumvented by appropriate design of both the protein and the activated polymer. Indeed, there are some examples of site-specific PEGylation, when activated mPEG was linked to a free thiol which has been engineered into the protein (Goodson and Katre, 1990; Benhar et al., 1994).

Another approach for a site-specific conjugation is to take advantage of the presence of a N-terminal serine or threonine which can be converted by very mild periodate oxidation to a glyoxylyl derivative (Fields and Dixon, 1968; Gaertner et al., 1992; Geoghegan and Stroh, 1992) and to functionalize the polymer with a complementary reactive function, the aminooxy group. An analogous method was shown to be straightforward for incorporating synthetic peptides into a protein backbone by introducing at the C-terminus of one fragment or peptide to be recoupled a hydrazide function (Gaertner et al., 1994) and a glyoxylyl group at the N-terminus of the other. In the same way, the aminooxy function will specifically react with the generated aldehyde group at the N-terminus of the polypeptide to form an oxime bond, which is more stable than the hydrazone bond. This approach has already been exploited for the site specific labeling of IL-8 at the N-terminus with an aminooxy-functionalized fluorescent probe (Alouani et al., 1995).

We report here the stoichiometrically precise, site-specific attachment of several functionalized polymers varying in size and shape, via an oxime linkage, to three different proteins: IL-8, G-CSF, and IL-1ra. Indeed, IL-8, as well as G-CSF after aminopeptidase digestion, present a N-terminal serine and threonine, respectively, which can be readily converted to an aldehydic group. A comparably reactive group was introduced at the N-terminus of Il-1ra by metal-catalyzed transamination (Dixon and Fields, 1972), although the conditions for this reaction are known to be potentially much more harmful to the protein. In all cases, the specifically introduced reactive carbonyl group reacts with the complementary aminooxy group of functionalized PEG (Scheme 1). The high homogeneity of PEG conjugates constructed in this way allowed us to explore the influence of the size and shape of the PEG polymer (linear or branched) on the

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[1] Abbreviations: mPEG, monomethoxypoly(ethylene glycol); IL-8, interleukin-8; IL-1ra, interleukin-1 receptor antagonist; G-CSF, granulocyte-colony stimulating factor; DCC, *N,N'*-dicyclohexylcarbodiimide; –OSu, *N*-hydroxysuccinimide ester.

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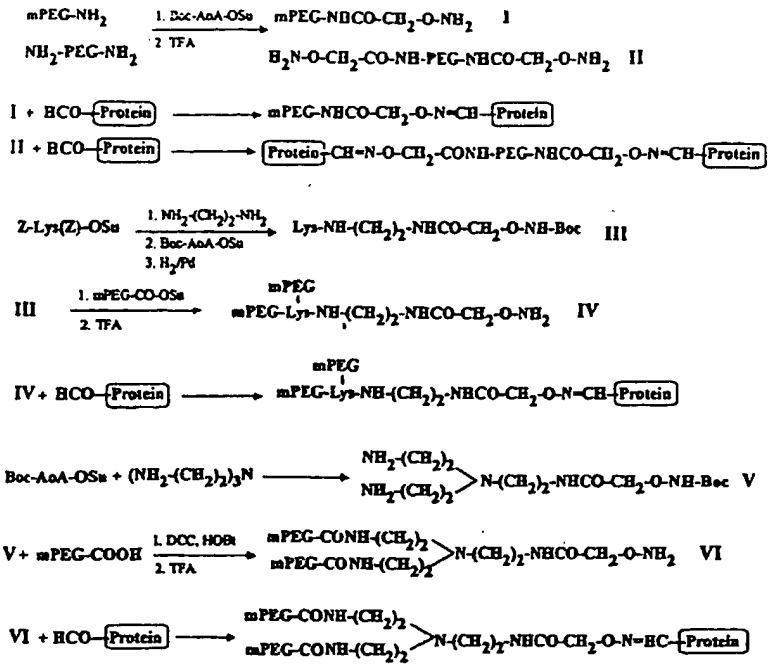
Scheme 1. Preparation of the Different Aminooxy Derivatives of PEG and Conjugation to the Reactive Carbonyl Group Generated at the N-Terminus of the Protein.



apparent Stoke's radius of the conjugates, on their *in vitro* activity, and on the blood circulation time in rats.

MATERIALS AND METHODS

Aminomethoxypoly(ethylene glycol) (NH_2-mPEG) of M_r 5000 was obtained from Shearwater Polymers (Huntsville, LA) and NH_2-mPEG and mPEG-COOH of M_r 10 000 and M_r 20 000 as well as NH_2-PEG_{20kD}-NH_2 from Rapp Polymere (Tübingen, Germany).

IL-8 was a gift from Glaxo IMB (Geneva, Switzerland). A mutant analogue of G-CSF was obtained from Zeneca Pharmaceuticals (Macclesfield, U.K.). This analogue known as TG119 is methionyl-[C17S; K16,23,34,40R; A111K; D112S]G-CSF. Il-1ra was a gift from Synergen, Inc. (Boulder, CO). Kidney microsomal aminopeptidase was from Sigma. $Na^{125}I$ was from Amersham, and female Wistar rats were from Iffa-Credo (L'Arbresle, France).

Synthesis of Linear (Aminooxy)acetyl-Functionalized Poly(ethylene glycol)(AoA-mPEG). ((Boc-amino)oxy)acetyl *N*-hydroxysuccinimide ester was prepared from aminooxyacetate (Fluka) (Vilaseca et al., 1993) and 144 mg (0.5 mmol) added to 1 g of NH_2-mPEG (0.2 mmol) dissolved in 4 mL of DMSO. The apparent pH was adjusted to 8.0 with *N*-methylmorpholine. After overnight stirring at room temperature, acylation was controlled by the quantitative ninhydrin procedure (Sarin et al., 1981) on a 10 μL aliquot of the reaction mixture. The acylated mPEG was then recovered by dilution with 5 volumes of water, followed by extensive dialysis against water and lyophilization. The Boc group was removed by dissolving the product in 10 mL of anhydrous TFA for 1 h at room temperature. After removal of the acid by rotary evaporation, the material was taken up in water, extensively dialyzed against water, and finally lyophilized yield 0.93 g (93%). The same procedure was used for the synthesis of AoA-NH-$mPEG_{10kD}$ and AoA-NH-$mPEG_{20kD}$ from the corresponding NH_2-mPEG, as well as for AoA-NH-PEG_{20kD}-NH-AoA from NH_2-PEG_{20kD}-NH_2.

AoA-$mPEG_{10kD}$ was analyzed by ^{1}H-NMR (200 MHz, $CDCl_3$), but only the PEG polymer signal (δ 3.62 ppm) was detected. However, the aminooxy end group could be characterized after derivatization with an aromatic ring. For this purpose, the polymer was reacted overnight with a 20-fold excess of benzaldehyde in a 0.1 M acetate buffer, counterion sodium, pH 3.6 containing 20% CH_3CN and the conjugation product isolated by semi-preparative HPLC, using a 35−75% B gradient over 20 min. The two signals at δ 7.4−7.6 ppm with intensities in the ratio 3:2, corresponding to aromatic protons, are consistent with the introduction of a benzaldehyde group through an oxime bond.

Branched Polymer $(mPEG)_2$Lys-NH-$(CH_2)_2$-NHCO-CH_2-O-NH_2, $(mPEG)_2$Lys-AoA. To 2.0 g (3.9 mmol) of Z-Lys(Z)-OSu were added 4 mL of ethylenediamine (60 mmol) and the mixture stirred for 2 h at room temperature. The coupling reaction was quantitative as checked by analytical HPLC on a C8 column using a linear gradient of 0−100% B over 50 min (t_R = 41.5 min instead of 48.5 for Z-Lys(Z)-OSu). The coupling product was purified by flash chromatography on a silica column equilibrated in $CHCl_3$/MeOH (9/1, v:v) and dried by rotary evaporation to yield 1.4 g of material. The product had the expected molecular weight, as determined by ESI-MS (calcd M + H, *m/z* 457.2; found *m/z* 458.0).

Z-Lys(Z)-NH-$(CH_2)_2$-NHCO-CH_2-O-NH-Boc was synthesized by adding 1.1 g of Boc-NH-O-CH_2-COOSu (3.8 mmol) to 0.9 g of Z-Lys(Z)-NH-$(CH_2)_2$-NH_2 (1.9 mmol) dissolved in 3 mL of dry DMSO and the apparent pH adjusted to 8−9 with *N*-methylmorpholine. The solution was stirred during 5 h at room temperature and diluted with 10 volumes of 0.1% TFA and the product purified on a preparative HPLC column 250 x 25 mm i.d. (Nucleosil 300A, 7 μm C8, Macherey Nagel, Oensingen, Switzerland) using a linear gradient from 40 to 80% B over 30 min with a flow rate of 8 mL/min. After lyophilization, the product (weight, 1.1 g; yield 90%) was characterized by ESI-MS (calcd M + H, *m/z* 630.2, found *m/z* 630.9).

The Z group was cleaved by catalytic hydrogenation. For this purpose, the material was dissolved in absolute

ethanol (50 mL) and acidified with 100 μL of CH_3COOH, 10% Pd/C (0.1 g) was added, and the mixture was hydrogenated in a hydrogenation apparatus overnight. The catalyst was filtered and the solvent removed by rotary evaporation. The product was purified on the same column that was previously used with a linear gradient from 0 to 50% B over 25 min. After lyophilization (weight, 600 mg; yield, 95%), the product was characterized by ESI-MS (calcd M + H, *m/z* 360.2; found *m/z* 361.8). The 2HCl salt was obtained by solubilizing the material in 1 mM HCl, freeze drying, and repeating the operation a second time.

Lys-NH-$(CH_2)_2$-NHCO-CH_2-O-NH-Boc·2HCl was then acylated with $mPEG_{5kD}$-COOH, $mPEG_{10kD}$-COOH and $mPEG_{20kD}$-COOH.

The PEG_{20kD}-COOH was preactivated by reacting 0.4 g (20 μmol) with *N*-hydroxysuccinimide (23 mg, 200 μmol) and DCC (21 mg, 100 μmol) in 3 mL of EtOAc. After overnight incubation at 37 °C, the precipitated dicyclohexylurea was removed by centrifugation and the supernatant treated with active carbon. After centrifugation, the PEG derivative was precipitated by cooling the solution to 4 °C. This precipitation was repeated three times to remove the excess of HOSu and DCC, and finally the material was washed with cold EtOAc and ether and dried in a vacuum desiccator. Two mg of the linker was then reacted with 210 mg of mPEG-COOSu (approximately a 1.5-fold excess over amino groups of the lysine derivative) in 200 μL of DMF. Acylation was complete after overnight stirring at room temperature. The solution was then diluted with 5 volumes of water, extensively dialyzed against distilled water, and finally lyophilized (180 mg).

The Boc group was then cleaved by dissolving the product in 2 mL of TFA for 1 h at room temperature. TFA was removed under vacuum, and the material was taken up in water, extensively dialyzed against water, and finally lyophilized to give the following derivative: $mPEG_{20kD}$-Lys($mPEG_{20kD}$)-NH-$(CH_2)_2$-NHCO-CH_2-O-NH_2.

A multivalent linker comparable to that described above was obtained by acylation of Tris−(2-aminoethyl)amine with 1 equiv of Boc-AoAOSu in DMSO. The monosubstituted derivative was isolated by HPLC and characterized by ESI-MS (calcd M + H, *m/z* 319.4, found *m/z* 320.6). The material was solubilized in 10 mM HCl, lyophilized, and further acylated with $mPEG_{5kD}$-COOH (1.2 molar excess over remaining amino groups) in DMF, in the presence of equimolar amounts of hydroxybenzotriazole and DCC. The solution was stirred overnight at room temperature and the fully acylated derivative purified after extensive dialysis against distilled water by ion exchange chromatography on DEAE-A25 and CMC-25 column and finally deprotected by TFA treatment to give the following derivative: ($mPEG_{5kD}$-CONH-CH_2-CH_2)$_2$-N-$(CH_2)_2$-NHCO-CH_2-O-NH_2.

Functionalization of the N-Terminus of the Protein and Conjugation with the Polymer. *1. IL-8.* The N-terminal serine of IL-8 (2−5 mg/mL) was oxidized with a 10-fold excess of sodium periodate in a 1% NH_4HCO_3 buffer, pH 8.3, in the presence of a 50 molar excess of methionine (a scavenger) during 10 min at room temperature. After 10 min, the reaction was stopped by the addition of a 2000-fold molar excess of ethylene glycol over periodate and the solution further incubated for 15 min at room temperature. The solution was then dialyzed against a 0.1 M AcONa buffer, pH 4.6. The oxidized protein solution was stored frozen if not used immediately. A 10-fold molar excess of a 10 mM aqueous solution of polymer was added to the oxidized protein, the pH adjusted to 3.6 with glacial acetic acid, and the mixture incubated for 20 h at room temperature. The conjugation product was purified from reagents by ion exchange chromatography on a Pharmacia Mono S column equilibrated in 25 mM AcONa buffer, pH 4.7 using a linear gradient from 0 to 2 M NaCl over 20 min (to eliminate the excess of polymer), followed by reversed phase HPLC on an analytical C8 column using a linear gradient from 30 to 65% B over 35 min (to separate unreacted IL-8).

2. G-CSF. G-CSF was concentrated to 4−8 mg/mL in a Tris·HCl 50 mM, 0.1% sodium lauroyl sarcosinate, pH 7.5 buffer and incubated with aminopeptidase (enzyme/substrate ratio 1/20, w/w) in the presence of $MgCl_2$ for 20 h at 37 °C. The commercial enzyme suspension was previously, after centrifugation, resolubilized in a 50 mM Tris·HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 2 mM PMSF, benzamidine and 2 mg/mL of aprotinin to prevent any cleavage of the protein by any contaminating proteolytic enzyme present in the commercial enzyme preparation. Aminopeptidase digestion was stopped by adding ethylenediamine tetraacetate (EDTA) at a 10 mM concentration, the solution dialyzed, at 4 °C, against a 20 mM Tris·HCl buffer, pH 8.0, and then against water, and the material finally purified by reversed phase HPLC on a 250 × 10 mm i.d. Nucleosil C8 column, using a flow rate of 3 mL/min and a linear gradient of 45−70% B over 15 min.

The material was then dissolved in 1% NH_4HCO_3, pH 8.3, containing 6 M guanidinium hydrochloride at 5 mg/mL and oxidation performed at room temperature with a 5-fold excess of periodate over 10 min. The reaction was stopped by the addition of a 1000-fold excess of ethylene glycol over periodate, and a 5 molar excess of AoA-$mPEG_{20kD}$ was added to the reaction medium. The solution was then diluted with 5 volumes of 0.1 M AcONa, pH 4.6 containing 6 M guanidium chloride and concentrated on a Centricon to the initial volume. The pH was then adjusted to 3.6 with acetic acid, and a further 5-fold excess of AoA-$mPEG_{20kD}$ was added to the reaction medium, which was then stirred overnight at room temperature. The conjugation product was isolated by reversed phase HPLC on an analytical C8 column using a 50−90% B linear gradient over 40 min with a flow rate of 0.6 mL/min.

3. IL-1ra. The concentrated protein solution (40 mg/mL) was diluted 10 times in a 2.5 M sodium acetate buffer, pH 5.5; $CuSO_4$ and glyoxylic acid were added to a final concentration of 2 mM and 0.1 M, respectively. The solution (final pH, 5.3) was then incubated for 30 min at room temperature and the reaction stopped by the addition of solid EDTA (50 mM, final concentration). The mixture was then extensively dialyzed against a 0.1 M AcONa buffer at pH 4.6 and the soluble fraction used for the conjugation reaction. The transaminated protein was incubated 40 h with a 10-fold excess of polymer in a 0.1 M AcONa buffer, pH 3.6 and conjugation followed by SDS−PAGE. The conjugation product was purified by hydrophobic interaction chromatography using a polypropyl aspartamide column (200 × 4 mm i.d., 1000A, PolyLC Inc., Columbia, MA), followed by gel filtration on a 600 × 7.8 mm i.d. BioSep-SecS 2000 Phenomenex column and ion exchange chromatography on a MonoQ column equilibrated in a 25 mM Tris·HCl, pH 7.6 buffer with a linear gradient to 25% of a second buffer containing 1 M NaCl with a flow rate of 1 mL/min.

***In Vitro* Biological Assay.** IL-8 activity was evaluated in a chemotaxis assay involving human neutrophils from healthy donors as already described (Alouani et al., 1995).

G-CSF Biological Activity. Granulocyte colony formation by peripheral blood progenitors isolated by cytapheresis was assayed in standard methylcellulose culture as described (Bender et al., 1994). The colony assay medium containing the cells, 10 ng/mL of IL-3 and IL-6, 100 ng/mL of SCF, 3 U/mL of EPO, and increasing concentrations of G-CSF was plated in duplicate in 35 mm Nunc suspension dishes and incubated at 37 °C in 5% CO_2, 5% O_2, and 95% relative humidity. Plates were scored after 14 days for colonies containing more than 50 cells.

The ability of modified IL-1ra to inhibit, in a dose responsive fashion, the PGE_2 production by dermal fibroblasts induced by IL-1β was measured after 72 h culture in the absence or presence of IL-1ra, as already described (Balavoine et al., 1986).

Pharmacokinetics of Native and Modified Proteins in Rats. IL-8 and its derivatives were iodinated under conditions, which have already been shown to retain full biological activity as determined by neutrophil chemotaxis (Grob et al., 1990). The specific activity of the radioiodinated IL-8 was 5−10 mCi/mg protein. ^{125}I-labeled IL-8 or its derivative was injected as a bolus (10 μg/kg, an amount limited by the potential toxicity of IL-8) in the tail vein of female Wistar rats weighing between 150 and 200 g. Blood samples were collected from the tail at 3, 7, 15, 30 min, 1, 3, 7, and 24 h. The samples were weighed to determine their exact volume, and their radioactivity was measured. Since a 2−3 min period is necessary to mix the injected material in the blood compartment, the sample collected at 3 min was considered as the initial concentration.

IL-1ra was also iodinated by the chloramine T methodology. Briefly, the protein was dialyzed against a 0.1 M Tris·HCl, 0.15 M NaCl, pH 7.0 buffer and concentrated to about 300 μL. This solution was then mixed with 500 μCi $Na^{125}I$ and 30 μL of a 1% (w/v) chloramine T solution prepared in the same buffer as that used for the protein. The iodination reaction was terminated after 90 s by the addition of 30 μL of a 5% (w/v) sodium metabisulfite solution and 25 μL of a 50% (w/v) KI solution. The entire reaction mixture was transferred on a GF5 column (Pierce) equilibrated in PBS and previously washed with the same buffer containing 0.1% BSA to separate the radioiodinated protein from free ^{125}I. The specific radioactivity thus obtained was in the range of 100−300 μCi/mg. The pharmacokinetics of IL-1ra and its derivatives was studied at a dose of 300 μg protein/kg under the same conditions as previously described.

Analytical Methods. SDS−PAGE was carried out on 20% Phast homogenous gels (Pharmacia) according to the manufacturer's instructions and the protein visualized by silver staining. IL-8, G-CSF, and IL-1ra were quantified by extinction coefficient $A_{1cm}^{0.1\%} = 0.89$, 0.86, and 0.93, respectively.

Analytical HPLC (250 mm × 4 mm i.d. Nucleosil 300A 5 μm C8 column) operated at 0.6 mL/min; 100% solvent A, 5 min then 2%B/min to 100% B; solvent A (1 g of TFA/1 L of HPLC grade water), solvent B (1 g of TFA/100 mL of HPLC water, made up to 1 L with HPLC grade acetonitrile). Electrospray ionization mass spectrometry (ESI-MS) was performed as already described (Gaertner et al., 1994).

RESULTS AND DISCUSSION

Coupling of Functionalized Polymers to the Proteins. *1. IL-8.* Oxidation of the amino terminal serine under conditions described was considered to be almost quantitative since two components could be identified ESI-MS corresponding to the glyoxylyl derivative derived from the methanol/water/acetic acid solvent used to inject samples (calcd, *m/z* 8350.7; found *m/z* 8350.8 ± 1.0) and especially the methyl hemiacetal derivative (calcd, *m/z* 8382.7; found *m/z* 8382.2 ± 0.6) while a unique mass spectrum was obtained for IL-8 (calcd, *m/z* 8381.7; found *m/z* 8380.9 ± 0.7). This result was further confirmed by the high coupling yield of the aminooxy functionalized polymers to the oxidized IL-8 (Figure 1). The SDS−PAGE characterization of mPEG−IL-8 samples shows also that a single homogeneous conjugate with increased molecular weight as compared to the parent protein is obtained, where the size is only dependent on the length of the polymer used and not on the degree of modification, as it occurred for attachment involving amino groups. The staining intensity of IL-8 and mPEG−IL-8 bands of analyzed reaction mixtures (Figure 1, lanes 1 and 3) and HPLC profiles (results not shown) allowed us to estimate the coupling yield to be about 80% in the case of AoA-$mPEG_{5kD}$ and AoA-$mPEG_{10kD}$. The lower coupling yields observed with AoA-$mPEG_{20kD}$ and AoA-$N(mPEG_{5kD})_2$ after 20 h of incubation (approximately 30%), could be significantly increased by prolonging the reaction period to 2−3 days.

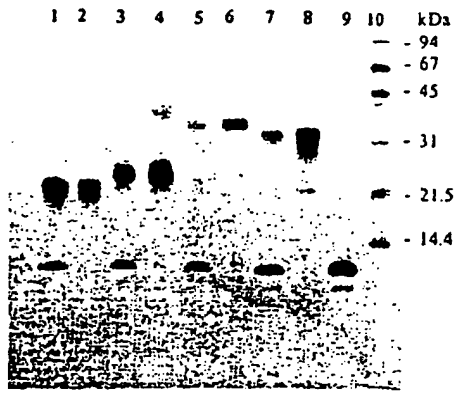


Figure 1. SDS−polyacrylamide gel electrophoresis of coupling between oxidized IL-8 and different functionalized mPEG polymers. Lanes 1, 3, 5, 7: result of 20 h of incubation of oxidized IL-8 with functionalized AoA-$mPEG_{5kD}$, AoA-$mPEG_{10kD}$, AoA-$mPEG_{20kD}$, and AoA-$N(mPEG_{5kD})_2$, respectively. Lanes 2, 4, 6, 8: purified $mPEG_{5kD}$−IL-8, $mPEG_{10kD}$−IL-8, and $mPEG_{20kD}$−IL-8 and $(mPEG_{5kD})_2$−IL-8. Lane 9: oxidized IL-8. Lane 10: protein markers from top to bottom, phosphorylase B (94 kD), bovine serum albumin (67 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean tyrpsin inhibitor (21.5 kD), lysozyme (14.4 kD). Unreacted PEG polymers were not visualized by silver staining.

The chemoselectivity of the ligation reaction allowed us to investigate the attachment of non standard PEG polymers, such as branched or bifunctional ones (Figures 1 and 2). The bifunctional derivative resulted in the formation of a protein dimer linked by a PEG chain ("dumbbell"). Although SDS−PAGE is not the most appropriate technique to characterize the effective size of each species, it can be clearly shown that constructs differing in topology but not in average molecular weight exhibit different apparent hydrodynamic radii. For example, in Figure 1, the behavior of $(mPEG_{5kD})_2$−IL-8 is much near to that of $mPEG_{20kD}$−IL-8 than to that of $mPEG_{10kD}$−IL-8. It is also worth stressing that the dimer construct was obtained in a single coupling step, even with a 5- or 10-fold excess of the bifunctional polymer over the oxidized protein.

2. G-CSF. The same approach was used in the conjugation of aminooxy-functionalized mPEG to G-CSF. But in this case, the N-terminal methionine had to be removed in a first step to reveal the threonine which will

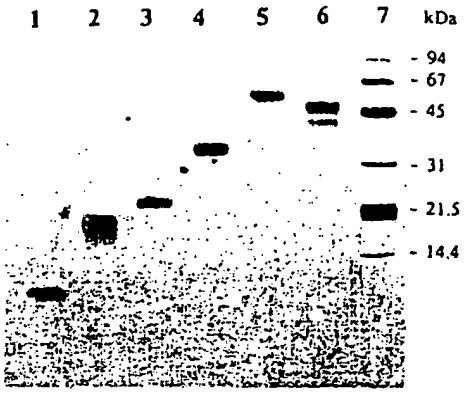


Figure 2. Characterization of IL-8 conjugates obtained with the different functionalized polymers after purification: lane 1; IL-8; lanes 2, 3, 4, $mPEG_{5kD}$–IL-8, $mPEG_{10kD}$–IL-8, and $mPEG_{20kD}$–IL-8; lane 5, $(mPEG_{20kD})_2$Lys–IL-8; lane 6, IL-8–PEG_{20kD}–IL-8; lane 7, protein markers.

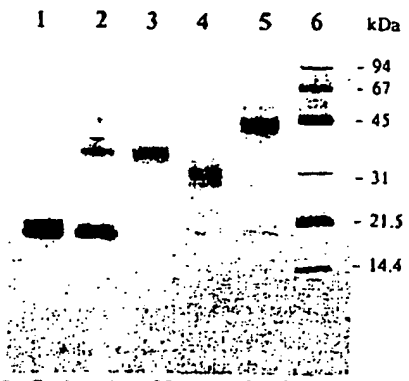


Figure 3. Conjugation of functionalized AoA-$mPEG_{5kD}$, AoA-$mPEG_{10kD}$, and AoA-$mPEG_{20kD}$ to IL-1ra: lane 1, IL-1ra; lane 2, coupling of AoA-$mPEG_{10kD}$ to transaminated IL-1ra after 48 h of incubation at room temprature; lanes 3, 4, 5, purified conjugates $mPEG_{10kD}$–IL-1ra, $mPEG_{5kD}$–IL-1ra, and $mPEG_{20kD}$–IL-1ra; lane 6, protein markers.

be the target of periodate oxidation. This could be readily achieved by enzymatic digestion with microsomal leucine aminopeptidase. This specific cleavage takes advantage of the presence of vicinal proline residue, which avoids any overdigestion of the protein substrate. This specific cleavage was confirmed by ESI-MS analysis of the digestion product, where only two compounds could be identified, the most important corresponding to the *des*-Met-G-CSF (calcd, *m/z* 18792.9; found 18792.1 ± 1.8) and the other to the native protein (calcd, *m/z* 18923.9; found *m/z* 18925.0 ± 2.8).

The oxidation could be performed under the same conditions as described for IL-8 and confirmed by mass spectrometry, but no coupling occurred without the addition of guanidinium chloride to the medium. This suggests that oxidation leads either to an unreactive species with no detectable mass change relative to the initial oxidized protein (by formation, for example, of an hemiacetal with a serine side chain) or to a conformational change leading to the steric inaccessibility of the carbonyl function. Under these conditions, the coupling yield did not exceed 20–30% and only the $mPEG_{20kD}$–G-CSF conjugate was synthesized at a larger scale for biological studies.

3. IL-1ra. The N-terminal Met-Arg-Pro– sequence of Il-1ra does not allow the use of the previously described method so that, in this case, selective modification of α-amino group was achieved by metal-catalyzed transamination. This method is likely to be much more general with regard to the N-terminal residue, but has the drawback of exposing the protein to a metal. Indeed, disappearance of the N-terminal amino group could not be assessed either by mass spectrometry, where several copper adducts only could be identified, or by electrophoresis, and so conditions chosen were close to those already described (Dixon and Fields, 1972). The appearance of the reactive keto group was detected by its ability to react with AoA-NH-mPEG. The transaminated protein was incubated for 48h with a 10-fold excess of polymer in a 0.1 M acetate (sodium), pH 3.9 at room temperature. $mPEG_{5kD}$–IL-1ra, $mPEG_{10kD}$–IL-1ra, and $mPEG_{20kD}$–IL-1ra were thus constructed and purified as shown in Figure 3. Owing to the fact that the coupling yield is lower than in the case of IL-8 and that some side reactions occur, isolation of the conjugation product required three purification steps and significantly decreased the overall yield of conjugate.

Biological Activities *in Vitro* and Pharmacokinetics in Rats. *1. PEG Conjugate of IL-8.* While IL-8 had an EC_{50} of 2 nM in the neutrophil chemotaxis assay (concentration corresponding to 50% of the maximal chemoattractant activity), $mPEG_{5kD}$ and $mPEG_{10kD}$ derivatives were shown to have an EC_{50} of 10 nM and $mPEG_{20kD}$, $(mPEG_{20kD})_2$Lys–IL-8, and IL-8-PEG_{20kD}–IL-8 an EC_{50} ranging between 20 and 40 nM.

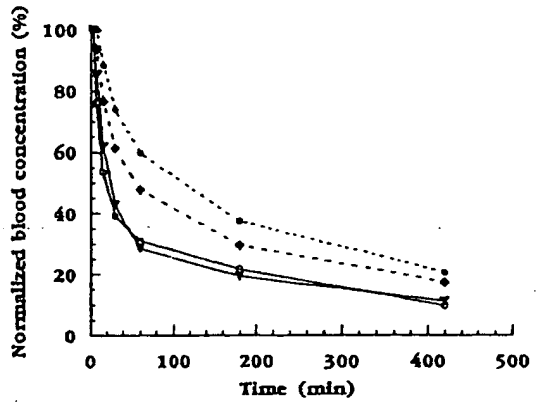


Figure 4. Pharmacokinetics of IL-8, $mPEG_{20kD}$–IL-8, $(mPEG_{20kD})_2$Lys–IL-8, and IL-8–PEG_{20kD}–IL-8. Rats were given a single injection of 10 μg/kg, i.e., IL-8 (○), $mPEG_{20kD}$–IL-8 (▼), $(mPEG_{20kD})_2$Lys–IL-8 (◆), and IL-8–PEG_{20kD}–IL-8 (●). Each determination is the mean value of four determinations with four animals.

The curves of relative blood levels for IL-8, PEG_{20kD}–IL-8, $(mPEG_{20kD})_2$Lys–IL-8, and IL-8–PEG_{20kD}–IL-8 given intravenously to the rats are compared in Figure 4. Conjugation of a single 20 kD chain to IL-8 has almost no influence on the pharmacokinetic curve, but the dumbbell and the $(mPEG_{20kD})_2$Lys derivatives have considerably increased area under the pharmacokinetic curve. A manual fit to these curves gives apparent first $T_{1/2}$ values of approximately 10 min for IL-8, 17 min for $mPEG_{20kD}$–IL-8, 30 min for IL-8–PEG_{20kD}–IL-8, and 60 min for $(mPEG_{20kD})_2$Lys–IL-8. No quantifiable differences were observed in the apparent second $T_{1/2}$ among all protein derivatives, but while less than 1% of the initially observed radioactivity remained in circulation after 24 h for IL-8, 6% of the initial amount was still present in case of $(mPEG_{20kD})_2$Lys–IL-8.

2. mPEG Conjugate of G-CSF. As shown in Figure 5, a dose–response curve was obtained for both *des*-Met-G-CSF and $mPEG_{20kD}$–*des*-Met-G-CSF. The biological activity of the *des*-Met-G-CSF derivative was slightly lower than that obtained with Neupogen which was used

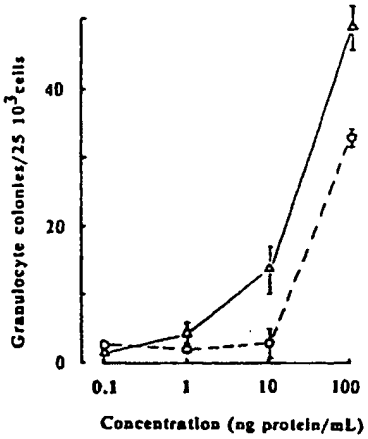


Figure 5. Comparison of *in vitro* biological activities of *des*-Met-G-CSF (△) and $mPEG_{20kD}$–*des*-MetG-CSF (○) using a methylcellulose culture medium to which were added IL-3 and IL-6 at 10 ng/mL, SCF at 100 ng/mL, and EPO at 3 units/mL using progenitor cells of human blood. 25 × 10^3 cells were deposited in each well, and G-CSF was added at increasing concentrations. Granulocyte colonies were scored after 14 days of incubation at 37 °C.

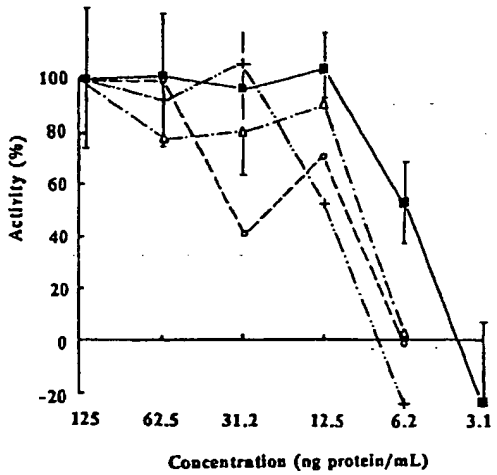


Figure 6. Inhibition of IL-1β-induced PGE_2 production by dermal fibroblasts in the presence of decreasing concentrations of IL-1ra derivatives. Each point is derived from a mean of three PGE_2 determinations, from which is substracted the mean of three determinations obtained when buffer only was used in place of IL-1ra. For clarity, error bars which are of the same order of magnitude as for IL-1ra (■) were not plotted for $mPEG_{5kD}$–IL-1ra (+), $mPEG_{10kD}$-IL-1ra (○), and $mPEG_{20kD}$–IL-1ra (△) derivatives.

as control (22 colonies/25 × 10^3 cells at 10 ng/mL), but the attachment of a $mPEG_{20kD}$ chain to the N-terminus resulted in a 2–3-fold decrease of the activity.

3. mPEG Conjugate of IL-1ra. As shown in Figure 6, the IL-1β effect is inhibited in a dose–responsive manner by all conjugates, and an IC_{50} value (concentration necessary to block 50% of IL-1β response) can be approximately determined: the values are approximately 13 and 9 ng/mL for $mPEG_{5kD}$– and $mPEG_{20kD}$–IL-1ra, respectively. The IC_{50} value for authentic IL-1ra was found to be about 6 ng/mL, and a similar profile was obtained for the transaminated derivative without conjugation of the polymer (results not shown).

The curves of relative blood levels for IL-1ra, $mPEG_{10kD}$–IL-1ra, and $mPEG_{20kD}$–IL-1ra given intravenously to the rats are compared in Figure 7. An

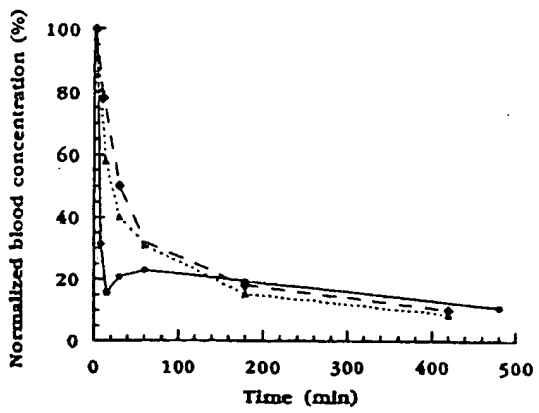


Figure 7. Pharmacokinetics of $mPEG_{10kD}$–IL-1ra and $mPEG_{20kD}$–IL-1ra in rats, compared with authentic IL-1ra. Rats were given a single injection of 300 μg/kg, i.e., IL-1ra (●), $mPEG_{10kD}$–IL-1ra (▲), and $mPEG_{20kD}$–IL-1ra (◆). Samples of blood were collected at 3, 10, 15, 30 min, 1, 3, 7, 12, and 24 h. Each determination represents the mean value of three determinations with three animals.

unexpected shoulder, reproducible from one animal to an other, could be observed in the IL-1ra clearance curve for blood samples recovered after 30 min, corresponding to a slight increase of circulating radioactivity which did not occur for other protein derivatives. A manual fit to these curves gives apparent first $T_{1/2}$ values of approximately 3 min for IL-1ra, 14 min for PEG_{10kD}–IL-1ra, and 20 min for PEG_{20kD}–IL-1ra. No detectable differences were observed in the apparent second $T_{1/2}$ amongst all three proteins, and in all animals less than 2% of the initialy observed radioactivity remained in circulation after 24 h.

It is clear from the results presented herein that by increasing the polymer mass or its molecular volume, either by enhancing its length or by adding multiple copies of the polymer at one site, one can increase the circulating half-life without marked loss of functionality. For these small proteins, where size is the rate-determing step in the *in vivo* clearance mechanism, only the size of the single polymer attached to the N-terminus of the protein will influence the pharmacokinetics of the conjugate, while with other conventional methods, the extent of modification of the different amino groups, which is less reproducible, has to be taken into account. Moreover, this method has the advantage of neither significantly decreasing the isoelectric point upon modification nor of introducing groups at locations likely to intefere with the binding properties of the target molecule. Pharmacokinetic data obtained with the 8 kD molecular weight protein IL-8 show that attachment of a single PEG_{20kD}-chain is not enough for significantly increasing the effective molecular size of the conjugate to overcome the cut-off point of kidney glomerular filtration (70 kD), whereas a comparable modification of the 14 kD IL-1ra results in at least 6-fold increase of the first half-life which is more consistent with a partial exclusion from kidney filtration. However, attachment of the bi-antennary $(mPEG_{20kD})_2$Lys-AoA construct markedly increased the circulatory half-life of IL-8, when compared to the single 20 kD polymer-modified protein.

The convenient functionalization of commercial amino-derivatized PEG polymers, or constructed multibranched structures described here, can provide a large panel of PEG derivatives varying in size and shape for a precise tuning of biological activity and plasma clearance of the target protein. Moreover, this approach can be extended

to other polymers such as dextran which can be monofunctionalized at its reducing terminus with a reactive amino group and further derivatized with an aminooxy function (H. Gaertner, unpublished results).

CONCLUSION

Results presented here demonstrate that proteins with an N-terminal serine or threonine are attractive targets for the site-specific attachment of aminooxy functionalized PEG polymers to generate improved biopharmaceutical proteins. This further extends the formerly described applications of this approach in protein backbone engineering (Gaertner et al., 1994) or immunoconjugate preparation (Werlen et al., 1994; Mikolajcjik et al., 1994). IL-8, which is an inflammatory cytokine, was used throughout this study only as a model protein which happens to have the appropriate N-terminal residue. The G-CSF modification demonstrates that even in the presence of a Met-Thr-Pro- N-terminal sequence, the threonine residue can be conveniently revealed by enzymatic digestion. Modification of IL-1ra without marked loss of activity shows that also metal-catalyzed transamination can be used for introducing a comparably reactive keto group at the N-terminus. This method is less restricted in terms of its N-terminal residues that are able to react, and it might therefore be a more general reaction.

Since in our approach the polymer is attached at a single site of the protein, it has to be of sufficient size to increase *in vivo* bioavailability, whereas in other methods such a result relies on the attachment of mPEG chains of relatively small size (5 kD) to several amino groups of the protein. The high coupling efficiency to oxidized IL-8 of PEG polymers, either linear or branched and varying in size from 5 to 40 kD, supports this hypothesis and prompts us to investigate the attachment of even larger multibranched polymers to the single reactive site of the protein via an oxime bond.

ACKNOWLEDGMENT

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Review article

PEGylation of cytokines and other therapeutic proteins and peptides: the importance of biological optimisation of coupling techniques

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Abstract

Polyethylene glycol (PEG) modification, PEGylation, is a well established technique which has the capacity to solve or ameliorate many of the problems of protein and peptide pharmaceuticals. It is one of the most important of the molecule altering structural chemistry (MASC) techniques and in many settings is enabling technology. The use of PEG as a linker molecule is also beginning to make a contribution to the production of exciting new products. We have previously reviewed the marked differences between methods of PEGylation and the surprising and dramatic impact of different coupling techniques (using different activated PEGs) on factors such as retention of bioactivity, stability and immunogenicity of the resulting PEGylated proteins and peptides. Numerous factors play a part in this variation: the presence or absence of linkers between the PEG and the target molecule; the nature and stability of the bond(s) between the PEG, linker and target; the impact of PEG attachment on surface charge; the coupling conditions; and the relative toxicity of the activated polymer and/or coproduct(s). These are not, however, the only sources of qualitative differences in PEGylated products. Our own experience whilst developing a linkerless PEGylation technique (i.e. one attaching only PEG to the target molecule), which we devised to overcome all the major problems of pre-existing PEGylation techniques, was that considerable modification of the prototype method and a process of 'biological optimisation' was required to achieve good results in terms of conservation of bioactivity. Biological optimisation has not, as far as we are aware, been systematically applied by other groups working in PEGylation. It is the term we use to describe an iterative process for examining and refining all the steps in the PEGylation process, *including* manufacturing the activated polymer, in order to achieve the best possible conservation of bioactivity and other beneficial features of the method. The application of this biologically optimised PEGylation technique, using tresyl monomethoxy PEG (TMPEG), to a variety of target proteins reveals, as outlined in this

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review, an exceptional ability to conserve biological activity of the target. This, and the benefit of adding nothing other than PEG itself (which has an excellent safety record), to the protein, as well as other manufacturing and practical advantages, makes the method ideal for the modification of cytokines and other therapeutic proteins.

Keywords: Polyethylene glycol; cytokines

1. PEGylation of protein and peptides

Polyethylene glycol (PEG) modification is a well established technique for the modification of therapeutic peptides and proteins. Although this class of pharmaceutical contains eight of the world's top 100 drugs in terms of sales, they are not ideal pharmaceuticals. Surmounting the problems posed by these agents is critical in order to exploit the plethora of novel peptides and proteins resulting from the genomics programmes which are 'prospecting' the body's own store of biologically active signalling molecules such as cytokines and chemokines. Surprisingly, for a single technique, PEGylation overcomes many of the pharmacological and toxicological problems of proteins and peptides. It also conveys pharmacoeconomic benefits, an important factor in today's harsh and competitive health care climate.

The main advantages of PEGylation, reviewed in [1–3], are applicable to a wide variety of peptide and protein therapeutic and diagnostic agents, some conventional pharmaceuticals and drug delivery systems such as liposomes. For proteins and peptides the benefits include:

(1) Markedly improved circulation time due to evasion of renal and/or cellular clearance mechanisms (both peptide and carbohydrate recognition systems are compromised);

(2) Reduced antigenicity and immunogenicity, including prevention of anaphylaxis;

(3) Improved solubility (insolubility at physiological pH can cause phlebitis at intravenous infusion sites);

(4) Resistance to proteolysis, including autodegradation of proteolytic enzymes;

(5) Improved bioavailability via reduced losses at subcutaneous injection sites, which can be extensive (60–80% of the injected dose);

(6) Reduced toxicity has been observed with a variety of types of adverse reaction [4]. This is to be anticipated for agents where toxicity relates to peak plasma dose (such as granulocyte-macrophage colony stimulating activity, GM-CSF [5]) because of the 'flatter' pharmacokinetic profiles achieved via subcutaneous administration of PEGylated proteins, also in cases where the toxicity relates to immune responses;

(7) Improved thermal and mechanical stability, which might be useful in the context of some of the more novel administration strategies;

(8) Easier formulation in materials used as the matrix in some depot (slow release) preparations.

In addition to the above benefits, the use of bi-activated PEG as a linker for targeting moieties, is also being explored [6]. X-PEG-Y constructs are an attractive alternative to fusion proteins because of the simplicity of their construction and because of the potential to build ranges of products in a combinatorial fashion. A good ligand can be used to target a range of useful therapeutic and diagnostic agents. Conversely a good toxic entity or chelator-bearing a radionuclide can be attached to many different ligands. This modular approach should dramatically reduce the cost of development, in comparison with custom-built single entities. Linkage of radionuclides to antibodies in particular has, with conventional attachment schemes, to be customised with each protein.

2. Problems of PEGylation methods

A wide variety of methods [7–26] has been developed to produce PEG-constructs. Although a modest number of useful products has been obtained, with the majority of methods of PEGy-

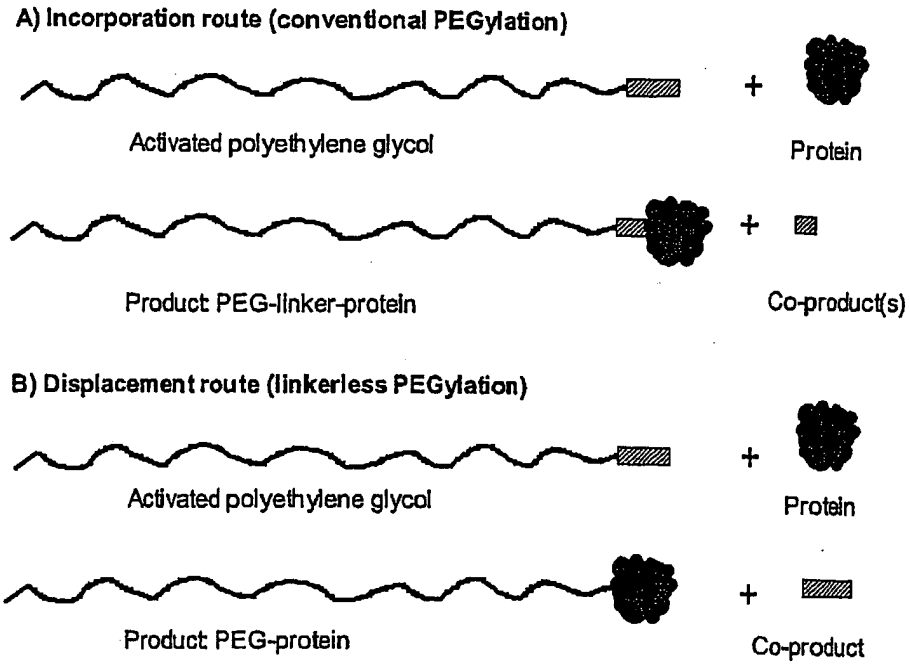

Fig. 1. Conventional versus linkerless PEGylation technology. With the 'incorporation route' part of the activating group (hatched) is included in the product. With the TMPEG method the N atom of an amino group (e.g. the NH_2 terminus or epsilon amino group of lysine) induces a nucleophilic displacement. The whole activating group is displaced and a stable secondary amine bond forms between the PEG and the target molecule. This displacement route leads to linkerless PEGylation.

lation, substantial reduction in bioactivity has been reported (typically 20–95%). Loss of bioactivity was particularly evident when applying these techniques to cytokines as opposed to enzymes with small substrates, but even the latter can show substantial reduction in activity (cf. PEG–superoxide dismutase, PEG–SOD, reported to retain only 46.8% of native activity [27]).

Initially, loss of bioactivity tended to be attributed to the mere presence of PEG chains. The early results with polymer modified enzymes and other proteins suggested a simple relationship between the number of PEG chains added to the protein and progressive loss of biological activity. We have demonstrated, however, that reduced bioactivity is often due to factors other than the mere attachment of PEG chains. In order to achieve different levels of PEGylation density on the surface of a target molecule, many factors vary other than the number of attached PEG chains. For example, the concentration of the activated polymer may be increased; more co-product will be released; incubation with the activated polymer and exposure to the harsh coupling conditions may be prolonged. Only when systematic studies applying different coupling techniques to the same protein were performed [3,28] did it become clear that a surprising degree of variation in retention of bioactivity, even from 100% to 0% of native activity, can be the result of simply changing the coupling method [3]. Thus the PEG chains themselves often contribute little or no obligatory loss of bioactivity over a considerable range of degrees of substitution.

Without these comparative studies, for any individual protein it is uncertain whether the degree of reduction in bioactivity observed is due to: (i) the attached PEG per se and the particular site(s) occupied; (ii) the degree of modification; (iii) adverse coupling conditions; (iv) the introduction of a linker between the PEG and protein (see below); (v) generation of harmful co-products; (vi) damage inflicted by the activated polymer. Each of these mechanisms might be influenced by the nature of the target protein being modified. The recommended coupling conditions, type of bond and co-product(s) of most major methods are illustrated in [3] and reviewed in [2].

In addition to the problem of reduced bioactivity, most methods for the activation of PEG result in part of the activating group being incorporated into the final PEG–peptide or PEG–liposome adduct (see Fig. 1 and reference [3]). These linkers can have several types of adverse effects:

(i) PEG itself is essentially immunologically inert [29], but the same cannot be assumed for the coupling moiety and there is experimental evidence for such groups being responsible for immunogenicity/antigenicity of PEG proteins. There are two routes of immunogenicity: stable coupling groups such as the triazine ring produced by the cyanuric chloride method are immunogenic in situ [30]. With unstable linkages, such as succinyl ester linkages, it is the residue remaining attached to the protein after the cleavage of the linkage which is immunogenic [31]. The evidence for the former is that antibodies to PEG–uricase cross reacted with PEG-SOD when both were made by the cyanuric chloride method, but failed to react with PEG–uricase prepared by the succinimidyl succinate method [30] which produces a different coupling moiety (see [3]).

(ii) Several coupling moieties contain labile bonds that can be cleaved enzymatically or chemically. In practice, coupling moieties with ester bonds appear the most susceptible [32]. Even in the absence of esterases, ester bonds are subject to slow hydrolysis [31] which poses storage problems. Amide bonds have been less well characterised but may presumably be cleaved where appropriate amidases are present (extra cellular fluid is a rich source of amidases). The carbamate bond, produced by several PEGylation methods [3] has been shown to liberate MPEG on serum exposure, more slowly than ester linked PEG, but more rapidly than amide linked PEG [32,33]. The mechanism of this release from the carbamate linkage is not yet clear, but has a curious inverse relationship to PEG chain length in some settings [34]. It should be noted that cleavage of labile PEG–target bonds does not necessarily lead simply to the loss of PEG, it can produce a modified protein bearing the residue of the linker (see (i) above).

(iii) Since many activating compounds are relatively toxic, coupling moieties derived from such groups cannot be assumed to be innocuous. This poses a regulatory question. Very little attention has yet been given to this point and virtually all linkers have no toxicological track record.

(iv) One coupling group at least (the triazine ring) is potentially reactive and was reported to cause crosslinking [11]. We have shown that the extent of crosslinking is variable, depending on the target modified [3].

Obviously, this constellation of problems is worth avoiding by turning to a linkerless PEGylation system. As indicated above, this is desirable from a regulatory point of view, since PEG has an excellent and long standing safety record, and the linkerless approach incorporates nothing other than PEG into the PEG–protein. However, linkerless methods of PEGylation are very unusual. Excluding the two linkerless methods developed by the present authors, we are only aware of one other method, first developed by Royer [35]. This uses neither the displacement nor incorporation routes of PEGylation illustrated in Fig. 1. PEG–acetaldehyde is the activated polymer in question and this couples to the protein to form an imine which is then reduced (usually with cyanoborohydride) to convert the linkage from an imine to a secondary amine, forming a terminal ethylene oxide unit in the process. This method, however, gives very poor conservation of bioactivity (Fig. 2).

Another problem area pertains to the ease with which PEG-adducts can be made and characterised. Where the activated polymer and/or co-products are toxic, biological assays cannot be performed without extensive, time consuming and costly purification steps. Examples of the impact of this toxicity on bone marrow culture and other assays are given in [3]. In an ideal method, all initial characterisation while 'fine tuning' the PEGylation reaction in the pre-clinical studies should be feasible using the PEG–protein product(s) without further manipulation.

Finally, some methods pose problems with respect to a variety of manufacturing issues: the lack of reproducible behaviour in coupling reactions; poor storage characteristics of the activated polymer; difficult or costly manufacturing; poor reactivity (requiring large excesses of activated polymer).

3. History of the TMPEG method

The tresylmonomethoxyPEG (TMPEG) method was based on a method devised by Nilsson and Mosbach for linking proteins to solid supports such as agarose, which was first published in 1981 [36]. This method attaches polymer primarily via NH_2 groups (either the amino terminus or lysine residues of proteins), but can also utilise free thiols. The linkage between the polymeric support and the target N atom was suggested to be a stable secondary amine bond and subsequent evidence supports this (see Appendix A). This linkage has several advantages. First, the polymer is linked directly to the target. Thus, in contrast to virtually all other polymer coupling techniques, no part of the group used to form the activated polymer remains between the polymer and the target molecule (Fig. 1). As mentioned above, such linkers have been the source of a variety of problems.

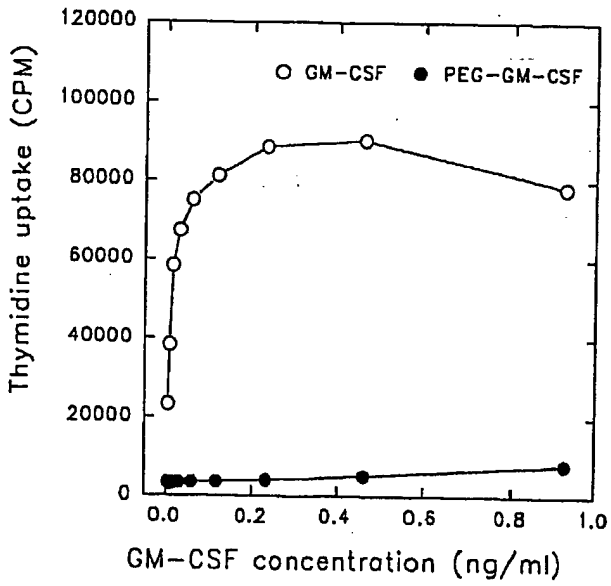

Fig. 2. Dose response curves for untreated GM-CSF (open circles) and PEG–GM-CSF (closed circles) produced by the PEG–acetaldehyde method of Royer et al. [35] as follows: GM-CSF at a final concentration of 50 μg/ml in a carrier protein solution at 1 mg/ml was incubated with PEG–acetaldehyde at 50 mg/ml and sodium cyanoborohydride at 11 mg/ml for 18 h at 37°C. The products were assessed for bioactivity as previously described [42] using a GM-CSF responsive cell line (TF-1) kindly supplied by Dr T. Kitamura.

An additional advantage of this linkerless coupling is that the secondary amine bond between the PEG and the protein, in contrast to several of the other bonds used in linker-based methods, conserves the ability of the N atom to which the polymer is attached to protonate. Thus conservation of surface charge at the attachment site is improved. Instances of surface charge change contributing to loss of biological activity include Veronese's observations on PEG–superoxide dismutase where the charge on a critical lysine group was found to be important for bioactivity [37].

One further advantage of tresyl as an activating group, not shared by the linkerless PEG–acetaldehyde method nor by many of the linker based methods [1–3], is that the coupling reaction can take place under very mild, physiological coupling conditions.

These benefits of Nilsson and Mosbach's activation method, and the recognition of the potential of this method to overcome major problems with pre-existing PEGylation techniques, led us to adapt it for activation of monomethoxyPEG (MPEG) to produce a reagent for the PEGylation of proteins and other substrates [22,38,39]. Considerable modification of our first prototype technique and a process of 'biological optimisation' was required to achieve good results in terms of conservation of bioactivity. 'Biological optimisation' has not, as far as we are aware, been systematically applied by other groups working in the field of PEGylation. It is the term we use to describe an iterative process for examining and refining all the steps in PEGylation process, *including* manufacturing the activated polymer, in order to achieve the best possible conservation of bioactivity and other beneficial features of the method. The application of this biologically optimised TMPEG technique to a variety of target proteins reveals, as outlined below, an exceptional ability to conserve biological activity of the target. Even delicate targets such as whole cells can be PEGylated. We have for example PEGylated red blood cells [40]. This was recently proposed as a source of 'universal blood' [41]. Application to other cell types may have relevance in some forms of transplantation, but this interesting field is beyond the scope of this review.

4. PEGylation of cytokines

4.1. Importance of biological optimisation

The typical development of a PEGylation method involves selection of an activation group; construction of a method for the production of the activated PEG and selection of appropriate coupling conditions, largely determined by the nature of the activating group. Thereafter, PEGylation of targets is perfected on a trial and error basis, usually examining a range of different degrees of substitution of the protein target. Different PEG chain lengths may also be evaluated. PEGylation usually results in statistical mixtures of PEG_n–protein products with a range of values of 'n' (occasionally only PEG_1–protein is produced). Typical examples are given in [42]. The bioactivity retention and other functions of the product are usually assessed on these mixtures, but individual members of the PEGylation series (PEG_1–protein, PEG_2–protein, PEG_3–protein etc.) may be assessed individually [43].

Most attention has historically been given to the optimisation of the degree of substitution with PEG chains and the selection of the best PEG chain length. Often, little consideration has been given to the early facets of method development. Our experience indicates, however, that virtually all steps in the process can have a surprising and very profound effect on the functionality of the product.

4.1.1. Selection of an activation group

This is of pivotal importance and the major contributor to between-method variation in conservation of bioactivity (see below).

4.1.2. Sources of PEG

Surprisingly, for such a simple polymer (PEG contains repeating ethylene oxide units and consists of a carbon–oxygen backbone with only hydrogen atoms attached), there are major differences in the quality of commercially available PEGs. Relatively early in its history, the field recognised the problem of heterogeneity of chain length (polydispersity) and of contaminating diol PEG in monomethoxyPEG (MPEG) preparations. Activation reactions contaminated by diol PEG give biactivated PEGs and the potential for crosslinking when producing PEGylated products. Diol PEG results from a side reaction early during polymerisation. Since, as a result, the polymer chain can grow at both ends, characteristically the diol PEG is often circa twice the molecular weight of the MPEG, hence easily identified and removed. There are, however, complex issues in the production of good quality PEGs. In our own experience we have even encountered MPEG samples which were essentially incompatible with the production of our own high performance activated PEG. A close collaboration with a provider of the raw material is essential for the best results and we must acknowledge the collaboration of Dr Nori Suginaka of NOF Corporation for helping us address the key quality issues. The results presented here and in our other publications on PEGylation use MPEGs manufactured by NOF Corporation (who produce a wide range of different sizes of MPEGs with low polydispersity and low diol content), supplied under licence by Shearwater Polymers, or low diol grade MPEG from Union Carbide.

4.1.3. Manufacture of the activated polymer

When manufacturing the activated polymer one might expect to be able to set reasonable criteria for yield and purity and leave the details of the process to the production chemist. In our experience this 'conventional optimisation' does not yield the best results. When we adapted Nilsson and Mosbach's activation method for solid supports to activate PEG, as we refined the method, the activated polymer was regularly checked in biological assays to determine whether the changes had beneficial or adverse effects on the functionality of the product. Retention of bioactivity and the toxicity of the reaction mixture products (containing the co-products and 'spent' excess TMPEG) varied markedly. We also checked for impact on issues relating to coupling reaction performance such as reaction rate, reproducibility and the rate of side reaction (see Appendix A). All of these factors are surprisingly susceptible to variation. Only by using systematic biological optimisation can the best results be obtained.

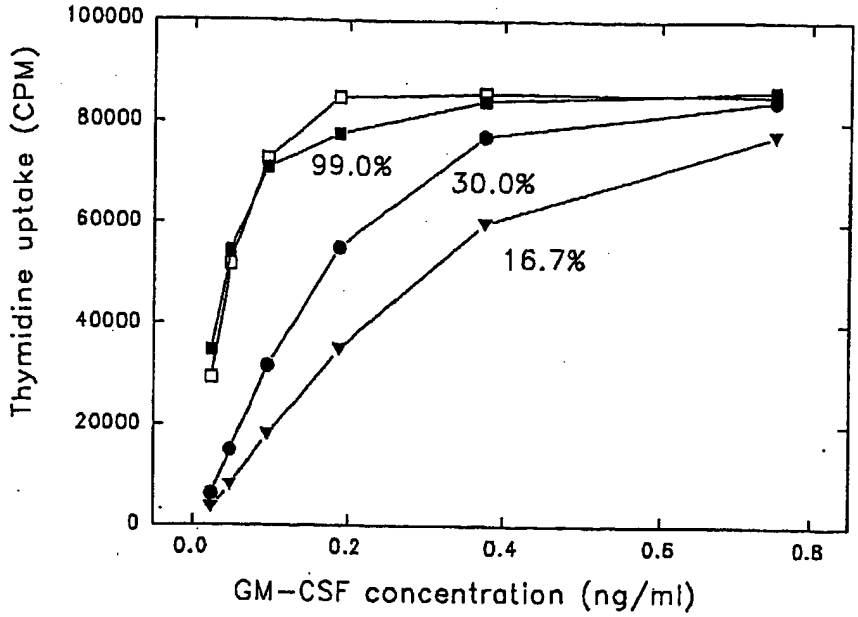

Fig. 3. Dose-response curves for sham-treated GM-CSF exposed to MPEG (open squares) and reaction products containing PEGylated GM-CSF made using different sources of TMPEG: the biologically optimised product (filled squares); and two conventionally optimised research grade catalogue materials from independent manufacturers (circles and triangles, respectively). The samples were assessed for bioactivity as in Fig. 2. The retention of bioactivity expressed as % that of the sham-treated controls is indicated for each PEGylated preparation.

Fig. 3 shows contrasting retention of bioactivity of PEG–GM-CSF, produced as previously described [42,44], but using TMPEG prepared by different manufacturing procedures. The reaction conditions used give predominantly one or two PEG chains and little residual unmodified material. The biologically optimised TMPEG sample gave 99% retention of bioactivity in the resulting PEG–GM-CSF with respect to an MPEG-treated control ($95.2 \pm 9.5\%$ mean $\pm$ S.E.M. in three independent experiments), whereas the PEG–GM-CSF derived from the TMPEG samples made by different manufacturing techniques from two independent suppliers and optimised by conventional criteria, had bioactivity of only 16.7 and 30% of control (13.7 ± 1.3 and $43.3 \pm 7.8\%$, respectively, in three independent experiments). These samples had similar TMPEG content with the biologically optimised sample having 93.9% TMPEG and the other two having 93.3 and 98.9% TMPEG respectively (reflecting the efficiency of the polymer activation step). TMPEG was assessed in the activated polymer preparations as % total mass, using reverse phase chromatography on a PLRP-S 100A 5 m column (Polymer Laboratories) with a 30–100% CH_3CN gradient and an evaporative mass detector (PL-EMD 960; Polymer Laboratories) at 85°C with gas flow at 5.5 l/min, to monitor the samples. Reactivity rates (assessed in partial PEGylation reactions using lysozyme) did not reveal differences capable of explaining this variation in retention of bioactivity (data not shown). These results are in agreement with previous reports from our own laboratory, where we observed significant improvement in the retention of bioactivity of PEG–GM-CSF as we optimised the manufacturing procedure for TMPEG over the years to improve our prototype modification of the Nilsson and Mosbach procedure [3,45].

During this process, we also found it was possible to substantially reduce the toxicity of the activated products. In GM-CSF colony cultures and cell line based assays, toxicity was essentially abolished and this resulted in our being able to assay the coupling reaction mixture containing the PEG–GM-CSF species without purification [3]. This is a valuable time saving, allowing the opti-

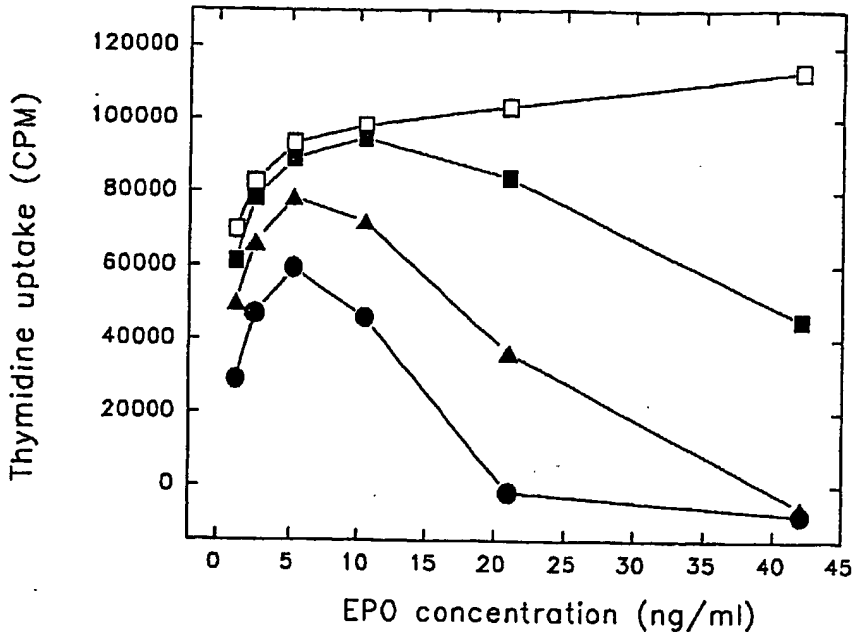

Fig. 4. Dose–response curves for sham-treated EPO exposed to MPEG (open squares) and reaction products containing PEGylated EPO made using different sources of TMPEG: a biologically optimised product (filled squares); and two conventionally optimised research grade catalogue materials from two independent manufacturers (circles and triangles, respectively). The samples were assessed for bioactivity as in Fig. 2 (the TF-1 cell line also responds to EPO).

mum PEG chain length and degree of substitution to be arrived at very quickly. These non-toxic reaction products can also be used directly in pharmacokinetic and other animal assays without further purification. Excess TMPEG in these reaction mixtures can either be quenched with a suitable nucleophile or allowed to hydrolyse to MPEG. Toxicity in erythropoietin (EPO) assays is a little more difficult to eradicate, but test and control which are superimposable over the majority of the dose response curve except the highest doses can still be achieved in samples with no significant loss of bioactivity produced by the biologically optimised method (see [3]). Again, the manufacturing procedure has a major impact. Fig. 4 shows assay of PEG-erythropoietin made by two TMPEG samples which had been produced by independent laboratories using 'conventionally optimised' manufacturing procedures. Toxicity is marked (note the progressive increase in departure from the sham-treated reference preparation at higher doses). The reference is erythropoietin exposed to MPEG which cannot attach to the protein. In these two samples, the reduced response at lower doses may be due to a combined effect of loss of potency in combination with the presence of inhibitory material. The biologically optimised TMPEG produced substantially less toxicity (note there was no toxicity for this material in the GM-CSF assay and no ad-

Fig. 5. Samples containing 1 mg/ml lysozyme and either 22 mM biologically optimised TMPEG (a) or conventionally optimised TMPEG (b) were analysed on a Beckman Instruments P/ACE 5010 capillary electrophoresis system fitted with a 27-cm (20 cm effective length), 75 μm I.D. amine-coated capillary (Beckman, catalogue no.: 477432) in a cartridge with a 100 × 800 μm window and a diode array detector (main spectrum continuously monitored at 214 nm). A 100-μl aliquot of the reaction mixture transferred to a 200-μl CE vial on the autosampler. The capillary was rinsed for 1 min with each of the following buffers: 0.1 M NaOH; amine regenerator solution (Beckman Instruments); 20 mM phosphate at pH 7.0 (running buffer). Electrophoresis was performed using 16 kV with optimum ramp time. The autosampler was set to repeat the analysis and the times of sampling were as follows: (a) front to back: 50.0, 39.2, 28.0, 17.3, 6.5 min; (b) 185.0, 141.7, 98.3, 55.3, 12 min. For ease of comparison each electropherogram is offset by 0.15 min and 0.005 AU from front to back (i.e. refer to the front electropherogram for the correct elution time).

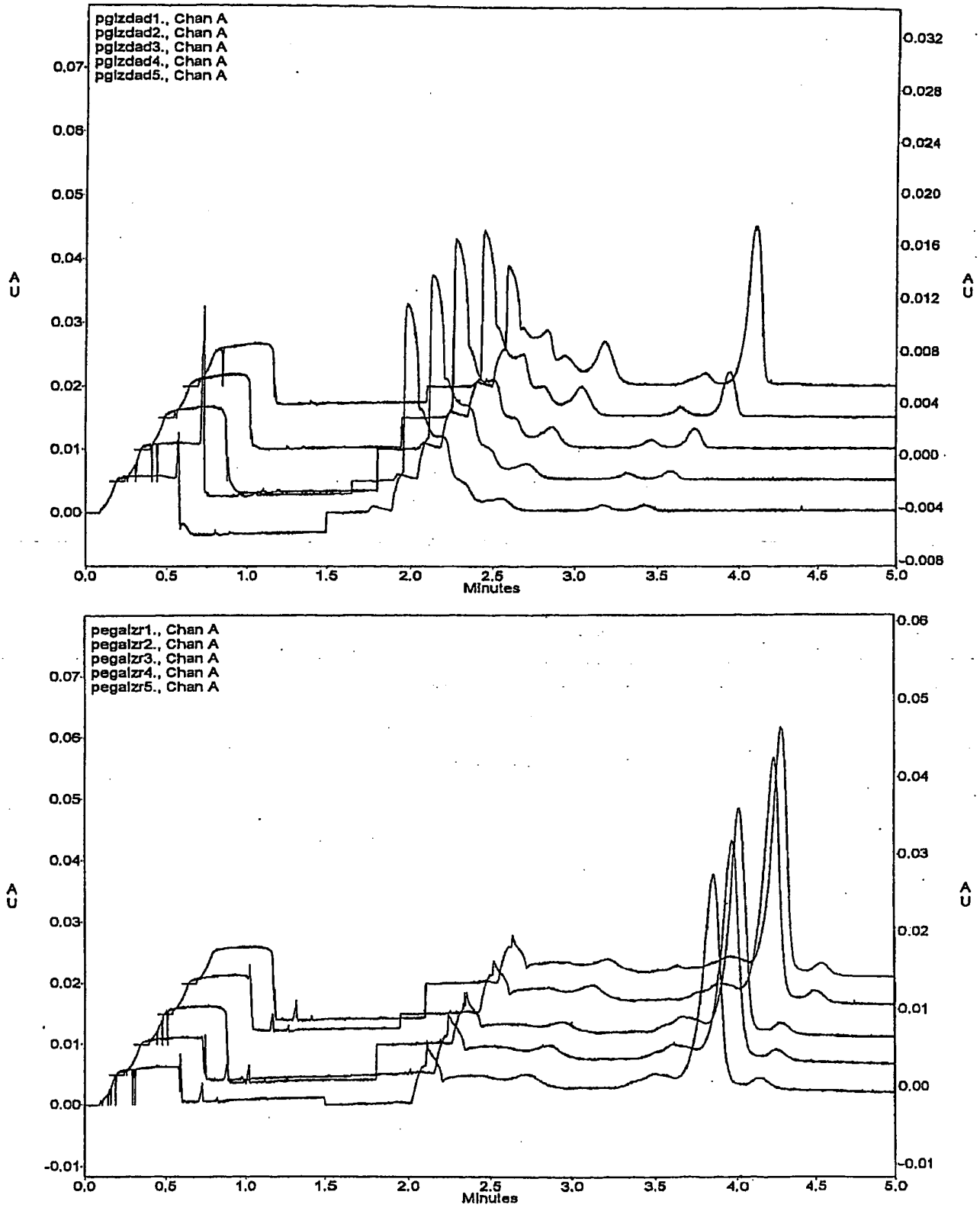

Fig. 5. (*Continued*)

verse effects in vivo during pharmacokinetic studies with the biologically optimised material).

One other surprising feature of the biologically optimised TMPEG manufacturing procedure is that reaction rates with proteins can be remarkably different from TMPEGs made by other procedures. Fig. 5a, b show results of capillary electrophoresis of two lysozyme PEGylation reactions: one where the TMPEG was made by the biologically optimised TMPEG (Fig. 5a) and another where the TMPEG was made using conventional optimisation criteria (Fig. 5b). Note that the time between samples is different in the two panels. Untreated lysozyme (not shown) showed only a large peak at ≈ 3.4 min and a small peak at 3.2 min. Dry TMPEG was dissolved in a solution of 1 mg/ml lysozyme (Fluka) in 20 mM phosphate buffer pH 7.0 (final concentration of 22 mM TMPEG) and sampled at the times shown. The biologically optimised TMPEG, showed some reaction products (eluting between 2.0 and 2.5 min) already evident in the sample analysed at 6.5 min and thereafter the unmodified lysozyme peak (extreme right peak at ≈ 3.4 min) declined rapidly until by 50 min this has almost disappeared. Of the product peaks, that at 2.5 min is prominent at 6.5 min and declines whereas the peak nearest the neutral position at ≈ 2.0 min progressively increases. This is typical of the formation of statistical mixtures of PEGylated protein products which show a trend toward higher degrees of substitution as the reaction proceeds. In contrast, the second TMPEG showed very little reaction within the whole of the 3-h 5-min observation period. Trivial explanations, such as differences in pH, were not responsible for this marked difference in reaction rate: a similar difference in reactivity was observed using size exclusion chromatography on Superose 12 and using a stronger coupling buffer than that designed for our biologically optimised TMPEG whose buffering capacity may be exceeded by TMPEGs made by other techniques (data not shown). Such profound difference in reaction rate can have a marked influence on the economics of a PEGylation method. The results also suggest that caution should be used when comparing statements about relative reactivity of different activated PEGs made by different laboratories.

4.1.4. Coupling conditions

The TMPEG method was selected because it enables the use of mild coupling conditions. The same is not the case with other methods and this can be a major contributor to poor performance. The TMPEG method requires rigorous control of coupling conditions including pH (ideally 7.0), temperature and duration of coupling.

4.2. Impact of PEGylation method

We have previously reported the remarkable impact of choice of PEGylation method on conservation of bioactivity and toxicity of reaction products [3] and will only therefore summarise the situation here. Figs. 6–8 contrast the fate of cytokines PEGylated with biologically optimised TMPEG and with other activated polymers. Results are expressed as correlation plots where the responses to a range of doses of the PEG–cytokine are plotted against the equivalent doses of unmodified cytokine. The analytical approach is illustrated in Fig. 6 using PEG–G-CSF as an example. To normalise the correlation plots, responses are expressed as % maximum response. These plots are easier to interpret than the raw dose response curves where it is difficult to assess the statistical significance of subtle departures between two dose response curves. The regressions (solid lines) and 95% confidence intervals (dashed curves) in Fig. 7 demonstrate that none of the PEG–cytokines shows statistically significant departure from native activity, i.e. none deviates significantly from the 45° line of equality (dashed line). In all these preparations the majority of the cytokine sample was PEGylated. The modal number of PEG chains varied, being lowest (one) with non-glycosylated EPO and higher with the other samples (which had mixtures of mainly one to three PEG chains in varying amounts). The attached PEGs were either $MPEG_{5000}$ or $MPEG_{12000}$.

In contrast (Fig. 8), the picture obtained with three other PEGylation methods (the cyanuric

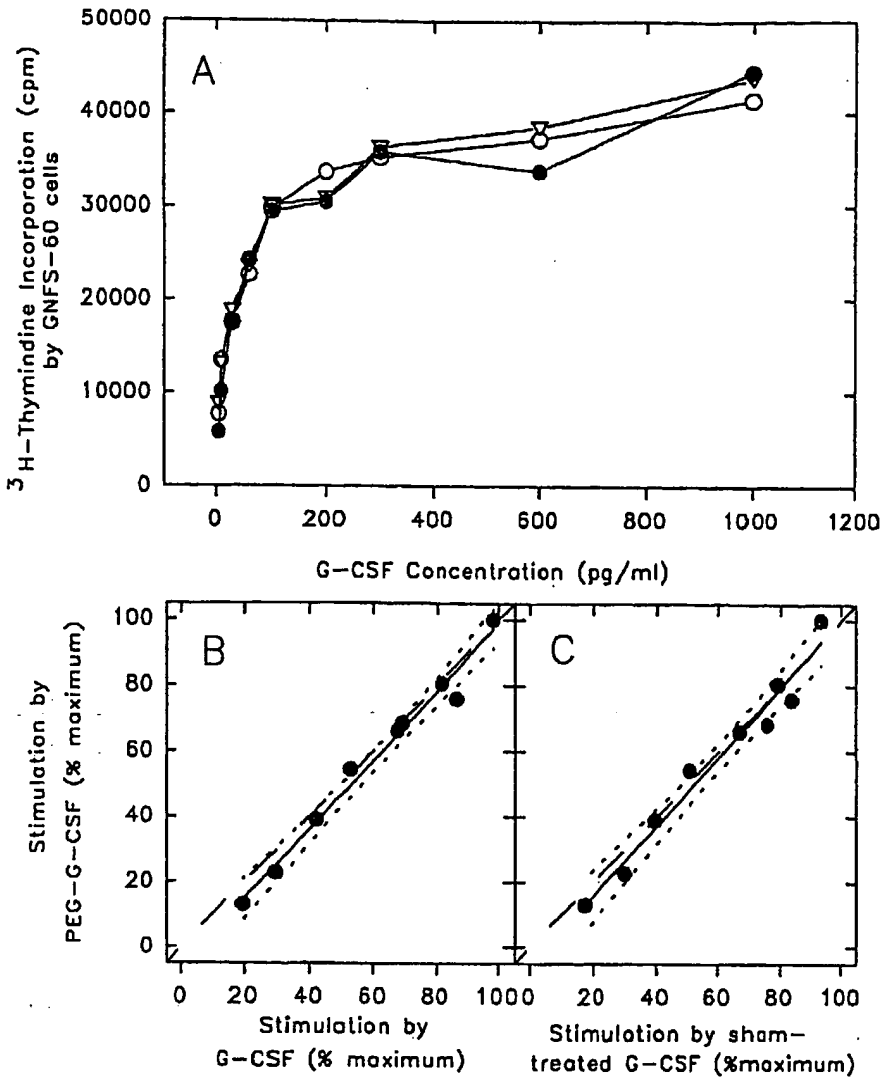

Fig. 6. Dose–response curves for G-CSF (open triangles), MPEG sham-treated G-CSF (open circles) and PEGylated G-CSF (filled circles) and corresponding correlation analyses. The PEGylated G-CSF contained 92% PEG_n–G-CSF with a modal value of 2 for *n* and was obtained using biologically optimised TMPEG 12 k. The samples were assessed for bioactivity essentially as in Fig. 2, but using the G-CSF responsive cell line GNFS-60 (American Tissue Culture Collection).

chloride, succinimidyl succinate and phenylchlorformate methods) was very different. All show statistically significant departures from the dashed line of equality. In these plots, upwardly concave departure from the equality line indicates simple loss of bioactivity (% retention of bioactivity is indicated in each panel) whereas downward curvature with maximum departure on the right is indicative of the presence of inhibitory material in the sample (see the upper and lower middle panels). Note that the middle panel results refer to colony assays which tend to be more sensitive to inhibitory effects than short lived thymidine uptake assays.

4.3. Impact of PEG chain length

Selection of the PEG chain length largely depends on the intended use of the protein. Fig. 9 shows glycosylated erythropoietin PEGylated by $TMPEG_{5000}$ and $TMPEG_{12000}$. The increased circulation time due to the attachment of the longer chain length is evident. The [^{125}I]EPO PEGylated with PEG_{12000} or with PEG_{5000}, was still de-

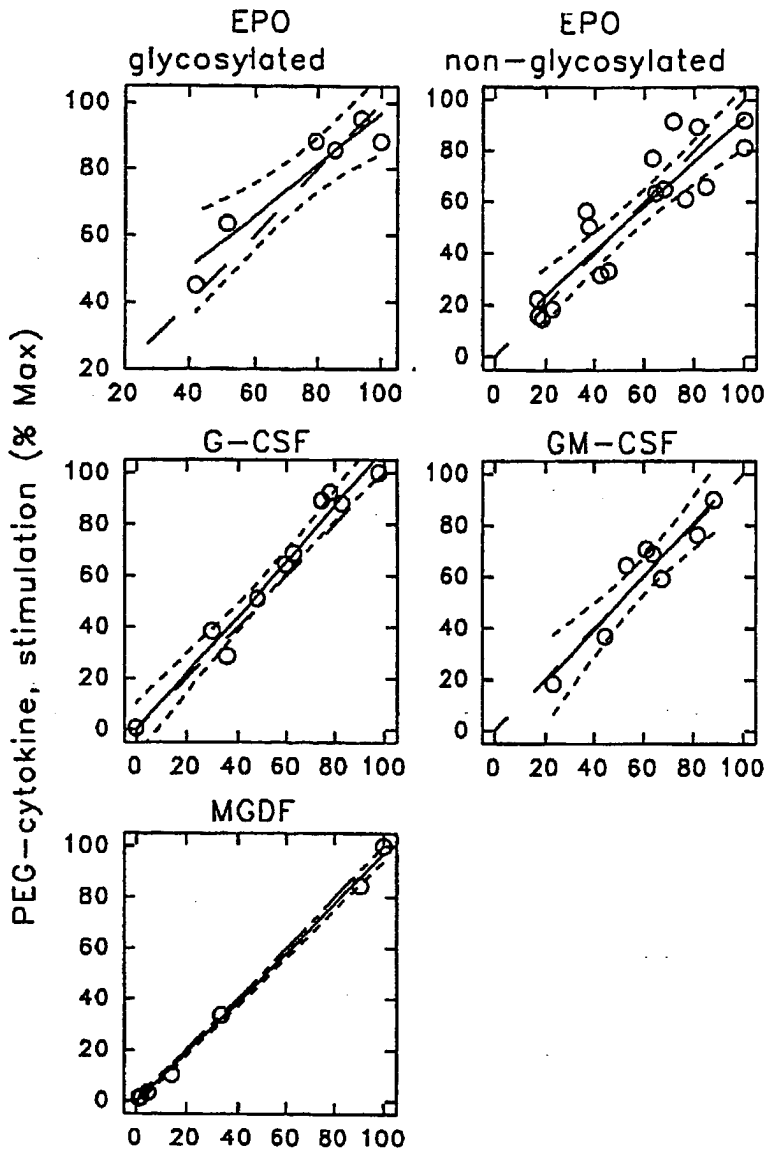

Fig. 7. Correlation analyses, performed as illustrated in Fig. 5, of PEGylated cytokines made using biologically optimised TMPEGs of either 5 k chain length (EPO, GM-CSF and MGDF) or 12 k (G-CSF). The dashed curves show the 95% confidence intervals for the regressions through the datum points. The 45° line of equality is indicated by a dashed line. Control samples, plotted on the horizontal axis, were sham-treated with the corresponding MPEG preparation in each case. The biological assays were performed as in Figs. 2–5 with the exception of MGDF which was kindly assayed by Dr A.R. Mire Sluis using thymidine uptake in a thrombopoietin-dependent cell line and glycosylated EPO which was tested in colony assays as for the EPO in Fig. 8.

tectable in the blood at 19 days post injection. The trend of increasing half life with increasing chain length has led some workers to use either very large linear PEGs [46] or branched PEGs [47]. There is, however, an advantage to using several PEG chains rather than a single large PEG in that, when the PEG–protein is eventually degraded, renal clearance of the PEG and residual amino acids can be rapid. PEG_{12000} is well below the renal threshold and should be readily cleared.

4.4. Impact of PEG chain number

Although increased circulation time is to be anticipated on the basis of escape from glomerular filtration due to the enhanced radius of the protein, in some proteins the number and size of

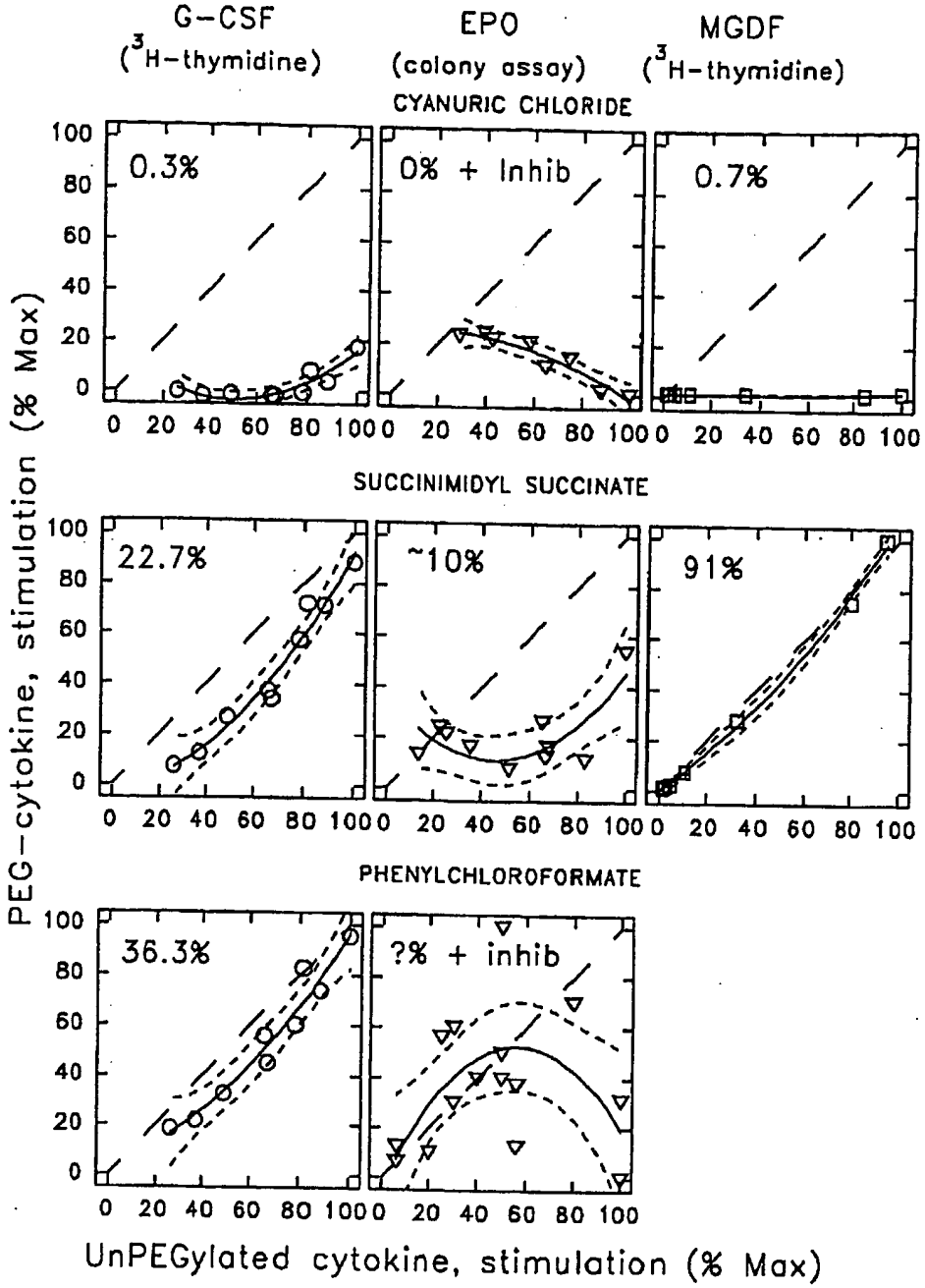

Fig. 8. Correlation analyses of PEGylated cytokines made using conventionally optimised activated 5 k PEGs (PEG–cyanuric chloride; PEG–succinimidyl succinate; PEG–phenylchloroformate, all obtained from Sigma UK). Control samples, plotted on the horizontal axis, were sham treated with 5 k MPEG. Thymidine uptake assays were used for G-CSF and MGDF as in Fig. 7 and erythroid colony cultures in methyl cellulose using normal human bone marrow cells were used to assay EPO as previously described [3]. The dashed curves show the 95% confidence intervals for the regressions through the datum points. The 45° line of equality is indicated by a dashed line. The values on each panel give the % retention of bioactivity. This could not be estimated in the case of PEG–EPO made by the phenylchlorformate method because of the presence of inhibition. The relative reactivity (with respect to TMPEG) of these activated PEGs [3] is insufficient to explain the marked losses of bioactivity seen here.

PEG chains to achieve prolongation of the circulation time is, somewhat surprisingly, unpredictable. Although Katre reported that, for IL-2, the changes in circulation time correlated well with the point at which the molecular size of the construct exceeded the renal threshold [48], the

same is not the case for PEG–GM-CSF. Using a PEGylation reaction series similar to those previously reported [42,44] in which GM-CSF is exposed to progressively increasing concentrations of TMPEG, GM-CSF preparations were produced in which the ratios of PEG_1–GM-CSF, PEG_2–GM-CSF, PEG_3–GM-CSF and more highly PEGylated series varied. Pharmacokinetic analysis of these samples showed that the first $MPEG_{5000}$ chain and possibly the second added to GM-CSF has the major impact on the circulation time with further PEG chains producing little additional effect. For example a reaction mixture containing 48.6% unmodified GM-CSF, 27.7% PEG_1–GM-CSF, 14.1% PEG_2–GM-CSF and only 3.1% PEG_3–GM-CSF showed a biphasic pharmacokinetic profile commensurate with the presence of both unmodified and modified species. The terminal elimination rate (likely to be due predominantly to the more slowly eliminating modified species) showed a 7.2-fold increase in blood half life with respect to control (11.7 versus 1.63 h). In contrast, a more highly PEGylated sample, obtained using a 30-fold higher concentration of TMPEG for the same duration of coupling (2 h), in which there was a much higher proportion of PEGylated species with more than one PEG chain, showed only a 10.3-fold increase in $t_{1/2}$ (16.8 h). This is somewhat surprising since the apparent molecular weight of PEG_1–GM-CSF is that of a globular protein of 41.1 ± 3.6 kDa on size exclusion chromatography on Superose 12 and 30 kDa by SDS-PAGE, i.e. apparently below the renal threshold. The discrepancy between Katre's and our own observations may relate to the handling of GM-CSF in the kidney where proteolysis by renal tubules degrades the protein to small peptides [49] and that PEG is known to convey significant protection against proteolysis.

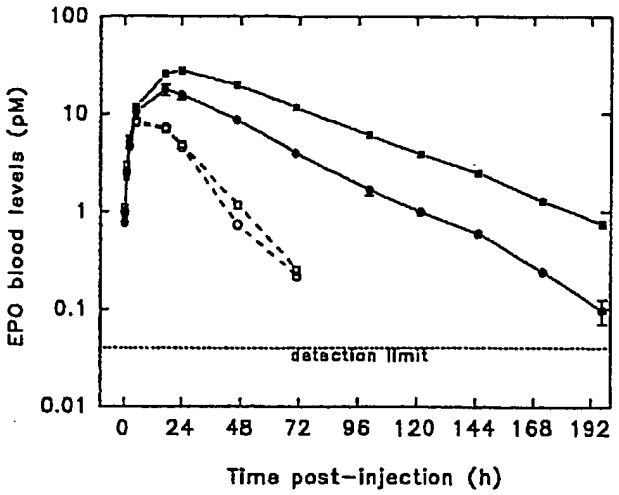

Fig. 9. The impact of attached MPEG chain length on circulation time. Glycosylated [^{125}I]EPO (Amersham UK) was PEGylated using either biologically optimised 5 k TMPEG (filled circles) or 12 k TMPEG (filled squares). The modal number of PEG chains was one, but PEG_2 and PEG_3 species were also present in both preparations. Control samples were sham-treated with the corresponding MPEGs (open circles and squares, respectively). Subcutaneous injections of 2.245×10^{-13} mol of control EPO and of PEG–EPO were administered to BALB/c mice. The $t_{1/2}$ values for the terminal linear portions of the elimination curves were 10 h for the control preparation and 20 and 29 h, respectively, for the 5 k and 12 k preparations.

When assessing PEG chain number it is important to appreciate that there are several potential pitfalls, especially if a single analytical technique is relied upon. Discussion of this point is beyond the scope of this review, but is published elsewhere [50].

5. Conclusions

Our experience with the PEGylation of cytokines has underlined the importance of biological optimisation of every step in the PEGylation procedure in order to achieve good conservation of bioactivity. Surprisingly, this even includes several steps in the manufacturing of the activated polymer. Other facets of performance such as toxicity of the reaction products and various properties of the activated polymers are also capable of substantial improvement using the biological optimisation process. Linkerless PEGylation techniques are rare, but attractive, in that they couple only PEG (which has an excellent safety record) to the target protein. The TMPEG method contrasts with the other linkerless method (the PEG–acetaldehyde of Royer et al. [35]) in that, whereas the former gives excellent conservation of bioactivity, the latter abrogates virtually all activity in the target. A new linkerless method is currently

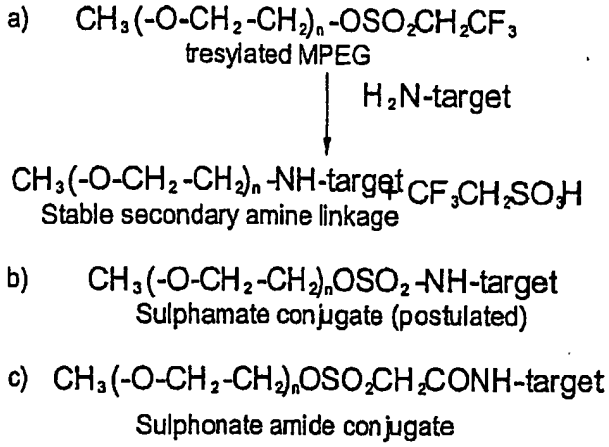

Fig. 10. (a) The coupling route resulting when TMPEG is exposed to protein at neutral pH. A nucleophilic displacement reaction results in all the activating group leaving and the formation of a direct polymer–target linkage without an intervening linker. (b) Proposed linkage (subsequently withdrawn). (c) Alternate linkage possible at high pH.

undergoing biological optimisation in our laboratories.

Appendix A

There is a great deal of confusion in the literature about the linkage actually formed by tresylated polymers. In 1994 Demiroglou et al. [51] published a statement that the linkage suggested by Nilsson and Mosbach was incorrect and proposed the linkage shown in the middle panel of Fig. 10. They based this statement on experiments using Sepharose 4B as the polymer, butylamine as the amino group bearing target and the observed elimination of fluoride at pH 13.3. F^- elimination was inconsistent with the proposed mechanism of Nilsson and Mosbach which was a nucleophilic displacement reaction with the N atom of the NH_2 group in the target displacing the entire activating group to yield the product and trifluorethane sulphonic acid (TFSA) as an innocuous co-product (TFSA does not itself break down to liberate F^-). Actually Demiroglou's statement ignored a caveat provided by Nilsson and Mosbach [36], who had themselves drawn attention to the possibility of fluoride elimination at high pH and had advised that such pHs be avoided.

Demiroglou's co-author Jennissen, later retracted their proposed conjugate structure [52] and reemphasised an observation cited in the original paper but not fully examined. Ohta et al., a year earlier in 1993 [53], had shown that some tresylated supports liberated only TFSA (the expected co-product for Nilsson and Mosbach's proposed linkage) and not fluoride, whereas others showed prominent fluoride elimination, inconsistent with the secondary amine. Thus the alternate reaction pathway, involving F^- elimination, was not only a consequence of high pH, but was also influenced by the polymer being used (Ohta used Sepharose 4B, diol silica and 5PW). Sepharose 4B showed particularly prominent F^- elimination. As Jennissen emphasised, it was therefore inappropriate for the linkage to be inferred from generalisations based on specific model coupling reactions. Interestingly, this is not the first occasion where the polymer element has been found to have a profound influence on coupling. Even the length of the polymer can be important (cf. the impact of PEG chain length on the stability of the carbamate linkage [34]).

In 1995 Gais and Rupert [54] published a proposed structure for the product of the alternate (F^- elimination) pathway (Fig. 10c). The linkage was obtained using *N*-butylamine and triethylene glycol (i.e. a much shorter polymer than PEG) at high pH. A subsequent publication on reactions of alkyl tresylates [55] further emphasised the pH dependence of the alternate pathway and confirmed the nature of the linkage as the sulphonate amide linkage shown in the bottom panel of Fig. 10c. The two pathways of formation of the alternate linkage are shown in Fig. 11. Monitoring the products of typical PEGylation reactions with protein targets (e.g. lysozyme) at physiological pH, we have not been able to find detectable levels of the sulphonate amide PEG–protein conjugate (Neale, Fisher, Delgado and Francis, manuscript in preparation). We have identified several key factors important in the lack of activation of the alternate pathway. These include: (1) avoidance of high pH; (2) factors relating to the manufacture of the activated polymer (TMPEG from different processes eliminates F^- at different rates); (3) the substrate can also reduce F^-

elimination (F^- elimination in the presence of substrate has been observed to be lower than elimination in buffer alone (Neale, Fisher, Delgado and Francis, manuscript in preparation)). High F^- elimination rates are not only undesirable with respect to generation of alternate product, but also in relation to the rate at which the activated polymer in the reaction mixture is inactivated. Fortunately, this problem is avoidable if all the above factors are understood and well controlled.

We have performed PEGylation reactions at high pH to examine the products of the alternate pathway and to investigate F^- elimination. These alternate pathway conjugates are readily detected by the fact that the linkage is labile at pH 9.0. Studies with lysozyme suggested that over half the linkages were alkali labile when PEGylation was performed at pH 9.0 (Neale, Fisher, Delgado and Francis, manuscript in preparation). If the conjugates are incubated at pH 7.0, the linkage is relatively stable (at least out to 24 h). Whether these linkages would be stable in vivo, however, is not certain since serum and body fluids contain a variety of amidases. We would therefore recommend that PEGylation is applied under the conditions where the alternate pathway is not activated. It should also be noted that the linkage also differs from the secondary amine in that it will not conserve surface charge.

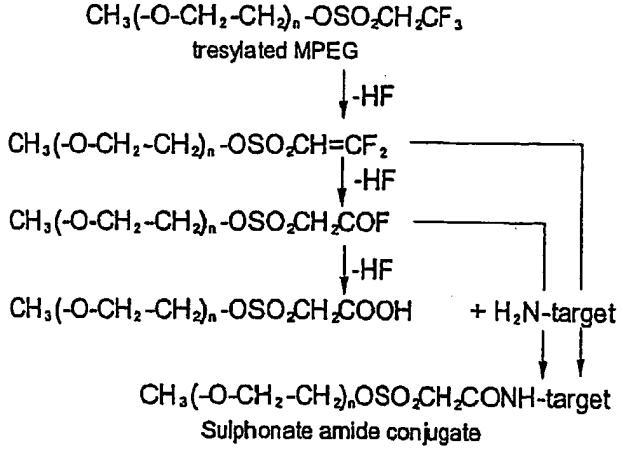

Fig. 11. The current proposals for alternate pathways of coupling. Fluoride elimination provides intermediates that react with NH_2 groups on the target to yield sulphonate–amide linked products. In the absence of target, elimination/hydrolysis yields the sulphonate acid.

In view of the caveat mentioned above, it is important not to extrapolate from one kind of PEGylation reaction to another. Thus when using unusual target molecules it would be prudent to examine this issue on a case-by-case basis. F^- elimination is readily monitored during PEGylation reaction using an F-electrode (F^- elimination is an obligatory step in the formation of the alternate linkage). In addition to the coupling reaction, during PEGylation, both hydrolysis to MPEG and TFSA and F elimination are also taking place and it should be noted that the balance between these two types of breakdown will not necessarily be indicative of the proportion of alternate linkages which would form in the presence of substrate. Importantly, significant variation in the rate of F^- elimination in buffer has been seen in samples of TMPEG made by different manufacturing processes. It should be noted that our failure to find evidence of alternate linkage formation at physiological pH applies specifically to our own manufacturing procedure and that the statement may not necessarily hold true for TMPEG made by different manufacturing processes.

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Controlled-Release, Pegylation, Liposomal Formulations: New Mechanisms in the Delivery of Injectable Drugs

K Rajender Reddy

OBJECTIVE: To review recent developments in novel injectable drug delivery mechanisms and outline the advantages and disadvantages of each.

DATA SOURCES: A MEDLINE (1995–January 2000) search using the terms polyethylene glycol, liposomes, polymers, polylactic acid, and controlled release was conducted. Additional references were identified by scanning bibliographies.

STUDY SELECTION AND DATA EXTRACTION: All articles were considered for inclusion. Abstracts were included only if they were judged to add critical information not otherwise available in the medical literature.

DATA SYNTHESIS: A number of systems that alter the delivery of injectable drugs have been developed in attempts to improve pharmacodynamic and pharmacokinetic properties of therapeutic agents. New drug delivery systems can be produced either through a change in formulation (e.g., continuous-release products, liposomes) or an addition to the drug molecule (e.g., pegylation). Potential advantages of new delivery mechanisms include an increased or prolonged duration of pharmacologic activity, a decrease in adverse effects, and increased patient compliance and quality of life. Injectable continuous-release systems deliver drugs in a controlled, predetermined fashion and are particularly appropriate when it is important to avoid large fluctuations in plasma drug concentrations. Encapsulating a drug within a liposome can produce a prolonged half-life and a shift of distribution toward tissues with increased capillary permeability (e.g., tumors, infected tissue). Pegylation provides a method for modification of therapeutic proteins to minimize many of the limitations (e.g., poor stability, short half-life, immunogenicity) associated with these agents.

CONCLUSIONS: Pegylation of therapeutic proteins is an established process with new applications. However, not all pegylated proteins are alike, and each requires optimization on a protein-by-protein basis to derive maximum clinical benefit. The language required to describe each pegylated therapeutic protein must be more precise to accurately distinguish each protein's differential pharmacologic properties.

KEY WORDS: polyethylene glycol, liposomes, polymers, polylactic acid, controlled release

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The development of new injectable drug delivery mechanisms has received considerable attention over the past few years. New drug delivery mechanisms can often provide benefits over standard formulations, particularly when the new formulation produces long-lasting drug concentrations.[1,2] Potential advantages include prolonged or increased pharmacologic activity, decreased adverse effects through lower peak drug concentrations and smaller fluctuations in drug concentrations, increased patient convenience, and improved quality of life.

Author information provided at the end of the text.

Although numerous methods exist for modifying the delivery of oral medications, alterations to injectable formulations have proven more difficult to develop. Peptides and proteins that have specific therapeutic uses (therapeutic proteins) are particularly attractive candidates for new injectable delivery mechanisms because they are hydrolyzed in the digestive tract, often making oral administration unfeasible. In addition, native proteins commonly have poor stability and a short half-life (thereby requiring frequent, multiple injections) that make them undesirable for use in chronic disease states. Accordingly, there is a demand for alternative delivery mechanisms that will optimize the therapeutic activity of peptides and proteins.

Novel drug delivery mechanisms are achieved either by novel formulation design of the drug or by modification of the drug molecule itself. In either case, a primary objective is to maintain the inherent therapeutic activity. Controlled-release formulations and liposomal preparations are examples of changes in drug formulation. Controlled-release formulations are designed to deliver the active agent in a continuous-release fashion, while liposomal preparations can be designed either for drug targeting or continuous release. In contrast, pegylation changes the molecular structure of the active agent to improve its pharmacokinetic profile while maintaining its intrinsic activity. This article provides an overview of recent developments in these novel injectable drug delivery mechanisms, outlines the advantages and disadvantages of each, and discusses specific examples of products that use these processes.

Injectable Controlled-Release Formulations

Injectable controlled-release formulations are designed to release drugs in a controlled, predetermined fashion and have been developed in an effort to provide more consistent pharmacodynamic effects and minimize adverse effects (characteristics that are particularly useful for drugs with a narrow therapeutic index). Most commonly, active agents are incorporated within a biodegradable and biocompatible polymeric matrix.

During the last 15–20 years, various biodegradable and biocompatible polymeric matrices, including microcapsules, nanospheres, and implants, have been developed. Microspheres are fine spherical particles containing active drugs. They are differentiated from nanospheres primarily by the size of the particle; microspheres have a diameter of less than approximately 1000 μm, while nanospheres are submicronic (<1 μm).[3] Microsphere systems contain either homogeneous monolithic microspheres, in which the drug is dissolved or dispersed homogeneously throughout the polymer matrix, or reservoir-type microspheres, in which the drug is surrounded by the polymer matrix membrane shell.[3,4] Monolithic and reservoir systems can also be combined. For instance, active drug can be dispersed within, or adsorbed onto, the polymer surface in a reservoir-type microsphere.

Biodegradable polymers can consist of either natural or synthetic materials that vary in purity. Natural polymers include polypeptides and proteins (e.g., albumin, fibrinogen, gelatin, collagen), polysaccharides (e.g., hyaluronic acid, starch, chitosan), and virus envelopes and living cells (e.g., erythrocytes, fibroblasts, myoblasts). Natural materials require cross-linking in the microencapsulation process, leading to the denaturalization of the polymer and the embedded drug.[3] As a result, synthetic polymers are most commonly used. Frequently used synthetic polymers include poly(α-hydroxy) acids such as polylactic acid (PLA), polyhydroxybutryic acid, and copoly (lactic/glycolic) acid (PLGA).[4] These compounds are biocompatible, lack immunogenicity, and have physical properties that permit them to be easily shaped (to control the bioerosion rate). These characteristics make synthetic polymers good candidates for extended-release delivery systems.

EFFECT OF DRUG FORMULATION ON PHARMACOKINETICS AND PHARMACODYNAMICS

Release of the active drug from a polymeric device is dependent on diffusion through channels and pores in the polymer matrix, diffusion across the polymer barrier, and polymer degradation.[3,4] In general, drug release is decreased as the density of the polymeric matrix increases. However, as polymer molecular weight is increased, proteins become impeded in their ability to be released from the matrix. Differences in hydrophobicity of PLA polymers and different ratios of PLGA copolymers can also have a large effect on release kinetics, as can the addition of solvents and other additives.[5] Degradation of polymers occurs by hydrolysis of ester bonds; the rate is dependent on the composition, molecular weight, and geometry of the polymer.[6]

Drug characteristics, such as the water solubility and crystallinity of the protein, can also affect the release kinetics of drugs.[5] Physiochemical drug properties are the most important factors controlling the release of highly water-soluble agents. For example, drugs with no basic group tend to release large amounts of drug at an early stage. Protein load can also affect release; increased concentrations of proteins generally result in a smaller proportion of the total amount of protein being released.

Polymer microsphere formulations of the highly water-soluble peptides, the luteinizing hormone-releasing hormone analogs (LHRH), have been evaluated; injectable leuprorelin acetate depot formulations and goserelin acetate implants are commercially available. The once-a-month depot formulation of leuprorelin acetate is a PLGA formulation, while the three-times-monthly depot formulation is a PLA formulation. The goserelin implant is also a PLGA formulation. The release behaviors of these water-soluble LHRH agonists are complex. After injection of leuprorelin acetate, serum drug concentrations show a large peak within the first day of administration, followed by a plateau within two days, and then stable but slowly declining concentrations over the remainder of the dosage interval.[7,8] In general, for the leuprorelin depots, the AUC is linearly correlated with the dose.[7,9] With the implantable goserelin preparations, serum concentrations are maintained for two to three months, resulting in a sustained suppression of serum testosterone secretion.[8]

More recently, polymer formulations involving larger, more labile, biologic proteins have been evaluated. However, since manufacturing procedures for these polymers use increased temperatures, surfactants, or aqueous/organic solvent interfaces, a potential complication is the loss of biologic activity through denaturation.[10] For example, in one study,[6] >99% of interferon beta activity was destroyed when standard heat-melt extrusion techniques for implant preparation were used (this problem was overcome by producing a microemulsion of precipitated protein in a solvent solution of PLGA).

Maintaining protein integrity during encapsulation and release is a difficult obstacle, particularly when proteins are in a hydrated state at high concentrations and at body temperature. Novel, biodegradable PLGA microsphere formulations are being developed in an attempt to overcome the problems of protein stabilization and encapsulation. These include human growth hormone,[10-12] thyrotropin-releasing hormone,[13] interleukin-2, and calcitonin.[14]

CLINICAL CONSIDERATIONS

Advantages and disadvantages of injectable polymer formulations are summarized in Table 1. The primary advantages are a prolonged duration of therapeutic effect due to less fluctuation in plasma drug concentrations. This has the potential to result in enhanced efficacy in situations in which the maintenance of steady-state plasma drug concentrations is important for therapeutic effect. Other potential advantages include increased compliance and improved quality of life. Disadvantages include the instability of some formulations and the difficulty in developing an acceptable release profile. An additional disadvantage of implantable formulations is that they must be inserted under local anesthesia.

Liposomes

Colloidal particulate carriers have attracted considerable attention as a method of delivering drugs.[15] Liposomes are the most commonly used colloidal vehicle; they are composed of a phospholipid bilayer that may act as a carrier for both hydrophilic and hydrophobic medications.[15,16] The unique structural properties of liposomes provide them with a number of desirable properties. They alter tissue distribution of the drug, thereby improving efficacy and reducing toxicity. Liposomes can be formulated in a wide variety of shapes and sizes and are composed of substances with low intrinsic toxicity, such as neutral phospholipids and cholesterol (Figure 1).[16]

EFFECTS OF LIPOSOMAL FORMULATION ON PHARMACOKINETICS

Liposomal formulations can affect drug pharmacokinetics by a number of mechanisms, including decreased volume of distribution (and a shift in distribution in favor of diseased tissues with increased capillary permeability), delayed metabolism, and delayed clearance.[16] The half-life of a liposomal drug depends on the size and composition of the liposome. Liposomes are cleared from the blood by the mononuclear phagocyte system, which is comprised primarily of Kupffer cells in the liver and fixed macrophages in the spleen.[16] In general, clearance is directly related to the size of the liposome, with larger molecules associated with a longer half-life. The addition of an amphophilic polymer such as polyethylene glycol (PEG) onto the surface of a liposome can slow the clearance of liposomes.[17]

Encapsulation of the anthracycline doxorubicin into a liposome has been shown[18] to substantially alter the pharmacokinetics of the drug. Compared with the standard formulation, liposomal doxorubicin has lower peak plasma concentrations, reduced volume of distribution, increased AUC, reduced clearance, and a longer half-life. The cardiotoxicity of anthracyclines is caused by localization of the drug in the cardiac tissue and appears to be correlated with high peak plasma drug concentrations.[16,18] Given the lower plasma concentrations associated with the liposomal formulation and the limited distribution of liposomes into the myocardium, liposomal doxorubicin may be associated with decreased cardiotoxicity.[16]

Table 1. Advantages and Disadvantages of Injectable Delivery Systems

System	Advantages	Disadvantages
Polymeric devices	long-lasting drug concentrations less fluctuation in plasma concentrations increased compliance and QOL	poor release profile instability of formulation need for local anesthesia (implants)
Liposomes	improved pharmacokinetics ($\uparrow t_{1/2}$, $\uparrow$ AUC, $\downarrow C_{max}$, $\downarrow V_d$, $\downarrow$ Cl) decreased toxicity passive targeting	sequestration into RES vascular "leak" difficulty in achieving long-term physiochemical stability activation of complement
Pegylation	improved pharmacokinetics ($\uparrow t_{1/2}$, $\downarrow$ Cl, $\downarrow C_{max}$) less fluctuation in plasma concentrations enhanced in vivo activity decreased toxicity increased compliance and patient QOL decreased immunogenicity increased physiologic and chemical stability	loss of activity with improper selection of PEG and/or pegylation

Cl = plasma clearance; C_{max} = maximum plasma concentration; PEG = polyethylene glycol; QOL = quality of life; RES = reticuloendothelial system; $t_{1/2}$ = elimination half-life; V_d = volume of distribution.

CLINICAL CONSIDERATIONS

Liposomes can be used to passively target the delivery of active drugs to diseased tissue. In diseases associated with increased capillary permeability (e.g., cancer, infection, inflammation), drug-containing liposomes concentrate in diseased tissue to a much greater extent than in normal tissue (where intact capillaries are impermeable to liposomes).[16] Once the liposomal drug is localized to the target tissue, unbound drug is slowly released by the liposome and taken up by cells of the target tissue.[16,19]

Potential advantages of using liposomes for the delivery of antibiotics include increased antibiotic concentrations at the site of infection, increased intracellular antibiotic concen-

trations, and reduced toxicity.[17,20] Not only can liposomes be used as carriers of antibiotics, but their ability to localize in the mononuclear phagocyte system can be used to target macrophage modulators, such as muramyl peptides or interferon gamma, thereby enhancing nonspecific host defenses.[17]

Liposomes also have potential applications in the delivery of drugs to specific regions and topical sites such as the lungs, peritoneum, eyes, and wounds or burns.[21,22] For example, the phospholipid particles contained in liposomes are similar in composition to lung surfactant, making them biocompatible with lung tissue. Considerations in the development of pulmonary liposomal drug delivery include the stability of the liposome preparation in bronchoalveolar fluid, the size of the liposome vesicle for deposition in select regions of the lung, and the altered pharmacokinetics of the drug molecule.[22] Pulmonary liposomal delivery of antioxidants is one potential application of this technology. The delivery of contrast agents (e.g., iodixanol) to the liver where liposomes are selectively taken up is another example of regional delivery.[23,24]

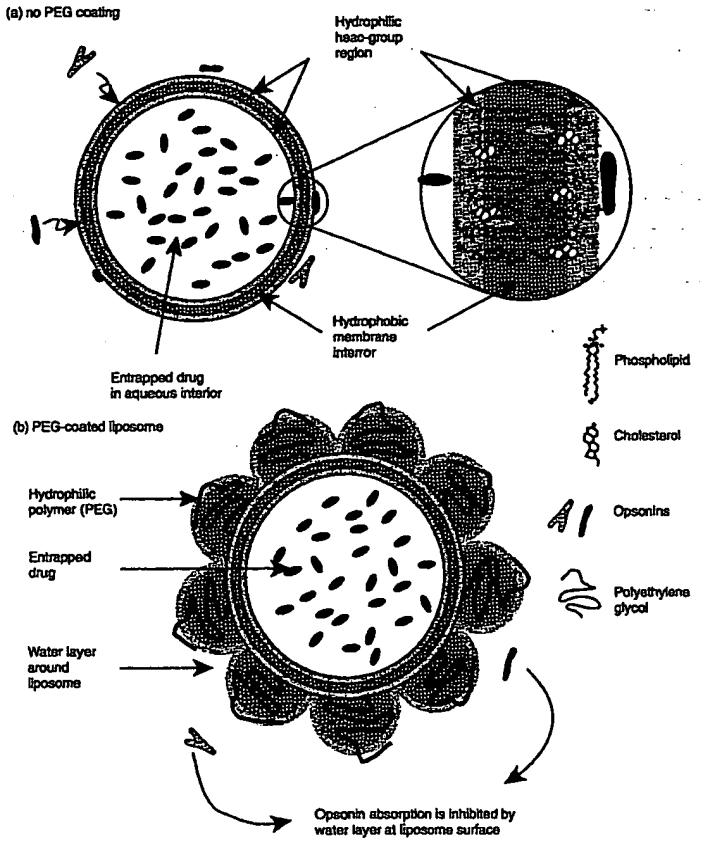


Figure 1. The structure of liposomal delivery systems. (a) Drug-containing liposomes in the absence of a polyethylene glycol (PEG) coating. Hydrophobic drug is present in the liposome membrane and hydrophilic drug is present in the liposome aqueous interior. Protein opsonins are shown absorbing to the "naked" liposome surface. (b) PEG-coated liposome with entrapped drug in the liposome interior. Protein opsonins have significantly less absorption to the liposome surface.[16]

EFFECTS OF LIPOSOMAL FORMULATION ON TOLERABILITY

Confining the drug to the vasculature via the use of liposomal carriers decreases drug distribution to normal vasculature (e.g., the heart), and therefore has the potential to decrease adverse effects. The use of liposomes may be particularly appropriate for drugs that have narrow therapeutic indices. For example, liposomal drug delivery systems have been extensively investigated in the delivery of antineoplastic and antiinfective agents.[17,25,26] The antifungal agent amphotericin B has been encapsulated into liposomes and bound to lipid carriers (e.g., lipid complex, colloidal dispersion) in an effort to reduce its toxicity and improve its therapeutic index.[17,25,27] Amphotericin B lipid complex is a concentration of ribbon-like structures of a bilayered membrane, while amphotericin B colloidal dispersion is composed of disk-like structures of cholesteryl sulfate complexed with amphotericin.[27] In clinical trials,[25,27] lipid formulations of amphotericin B have been shown to decrease the nephrotoxicity associated with the drug and decrease the incidence of acute reactions such as chills, rigors, and nausea. Although the lipid formulations may be associated with a decrease in antifungal activity,[25] they appear to produce an improved therapeutic index compared with standard amphotericin B formulations,[27,28] which is an important advance for patients who have experienced intolerance or failure with conventional amphotericin B therapy and have been successfully treated with these lipid formulations of amphotericin B.

LIMITATIONS OF LIPOSOMAL FORMULATIONS AND FUTURE DIRECTIONS

Despite the potential of liposomal drug delivery, the technology has a number of limitations (Table 1). These include the extensive sequestration of liposome particles in the liver, spleen, and reticuloendothelial system; the tendency of liposomal formulations to leak drug while in circulation; and the difficulty in achieving long-term physiochemical stability.[18,26] In addition, liposomes have a well-documented ability to activate the complement system.[29] This results not only in an enhanced clearance of liposome vesicles, but has the potential to cause a number of cardiovascular and hematologic adverse effects through the liberation of C3a and C5a, proteins involved in the complement cascade.

Second-generation liposomes are currently under evaluation in the hope of overcoming the limitations of earlier liposomal formulations. These newer formulations include the use of pegylated liposomes and immunoliposomes.[15,18] Modifying liposomes with PEG can

further prolong the blood-circulating time of liposomes. Compared with conventional liposomal preparations, pegylated liposomes are characterized by a slower plasma clearance, increased half-life, and a reduced volume of distribution. Pegylated liposomes are less extensively taken up by cells of the reticuloendothelial system and have a reduced tendency to leak drug while in circulation.[18,30] Pegylated liposomes also have been shown to have enhanced passive targeting of tumors.[30,31] Pegylated liposomal doxorubicin has been evaluated for the treatment of a variety of solid tumors, including ovarian and breast cancers and AIDS-related Kaposi sarcoma,[18,32,33] and has recently been approved in the US for treatment of ovarian cancer refractory to other first-line therapies. Immunoliposomes are designed to actively target solid tumors by attaching monoclonal or polyclonal antibodies to the surface of the liposome.[15,34-36] Such agents have the potential to transfer large numbers of drug molecules to an individual target site and have been shown to have antitumor activities similar or greater than native drug.

Pegylation: An Established Process with New Applications

Modification of drug molecules with PEG is a well-developed process aimed toward improving the delivery of injectable drugs and reducing adverse effects. PEGs are polymers comprised of repeating ethylene oxide subunits with two terminal hydroxyl groups that can be chemically activated. PEG molecules come in a number of different configurations (Figure 2).[37] PEG chains include linear and branched structures in which one or more PEG chains are joined with linkers such as lysine or triazine. Pegylation reagents are used to make PEG reactive toward amines, sulfhydryl groups, and other nucleophiles of the therapeutic molecule.[38] The site-directed pegylation of proteins is shown in Figure 3.[39] PEGs may be attached to a protein at a single site or at multiple sites.

EFFECTS OF PEGYLATION ON MOLECULAR PROPERTIES

PEGs are generally covalently linked to proteins; their addition may alter the properties of the protein in a number of ways. Pegylated biomolecules typically have different physiochemical properties than those of the parent drug molecule. The process of pegylation may alter the drug molecule; for example, by including conformational changes, steric interference, and changes in electrostatic-binding properties. The key to generating pegylated proteins with important therapeutic action is the optimization of the process to produce a therapeutic drug with the desired pharmacologic characteristics. These changes have the potential to result in enhanced pharmacokinetic and pharmacodynamic properties for PEG-modified proteins compared with unmodified proteins.[40] However, a PEG chain that is insufficient to protect the molecule offers no advantage to the parent molecule, while the use of too large a PEG conjugate and too many PEG conjugates attached can result in decreased biologic activity. Since branched-chain PEGs attach at single or fewer sites than do linear PEGs, branched PEGs may be less likely to interfere with the biologic activity of the native molecule than would the attachment of multiple small linear-chain PEGs.

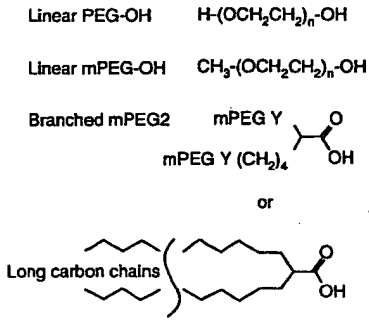


Figure 2. Chemical structures of polyethylene glycol (PEG) molecules.[37]

It has been demonstrated that there is a direct relationship between the mass of the PEG, circulating half-life, and AUC of the therapeutic molecule.[41] However, attachment of large PEGs or multiple sites of pegylation can also result in a decrease in in vitro bioactivity because there is an increased chance of PEG attachment occurring at receptor-binding domains.[37] Depending on the relative predominance of these opposing effects, pegylation can either increase or decrease in vivo activity. Due to this balance between protection from degradation and interference with bioactivity, it is essential that the pharmacodynamics of a pegylated molecule be carefully examined and optimized for each agent. PEG moieties may also interfere with other receptor-binding sites such as those that affect receptor-mediated drug interactions. The incidence or magnitude of established drug interactions, therefore, may potentially be altered by this phenomenon.

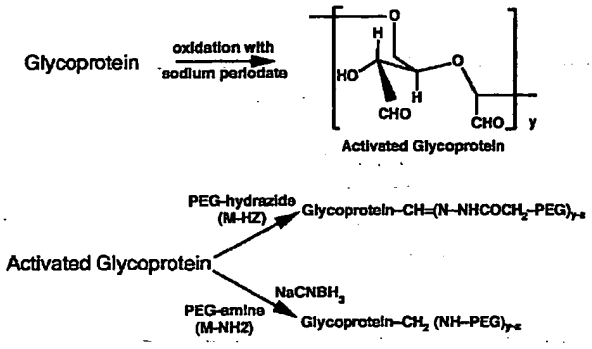
Pegylation is particularly useful for the modification of therapeutic proteins and peptides (e.g., endogenous proteins and peptides used for therapeutic purposes) that are required for sustained periods of time; these conjugates have generated much research.[42] The poor stability, short half-life, decreased clinical potency, and immunogenicity of native proteins preclude or limit their administration in an unmodified form. The rate of clearance of these molecules depends on the ionic charge and size of the molecule and the presence of cellular receptors. Modification with PEG may produce improved physical and thermal stability, protection against enzymatic degradation, and increased solubility.

CLINICAL CONSIDERATIONS

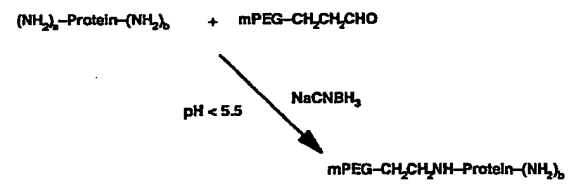
The physiochemical changes that may be produced by pegylation result in a number of potential clinical advantages (Table 1). These include enhanced pharmacologic activity, increased half-life, improved safety/tolerability, reduced immunogenicity, and increased patient compliance

and quality of life. Increased half-life has the potential to increase pharmacologic activity and therapeutic efficacy, which is important in particular clinical cases; for instance, in viral infection in which viral replication and resistance become an issue. Increased half-life is related to a number of mechanisms, including decreased renal clearance, decreased intracellular clearance, and increased resistance to proteolytic degradation.[1,40] Increased half-life also allows dosing intervals to be increased, resulting in improved patient convenience and enhanced quality of life. Avoiding high peak drug concentrations (associated with the frequent high doses often required with short half-life therapeutic proteins) may also decrease adverse effects associated with these peak drug concentrations. In addition, protein pegylation tends to decrease immunogenicity, presumably as a result of steric hindrance that prevents the immune system from recognizing the therapeutic protein as foreign.[2]

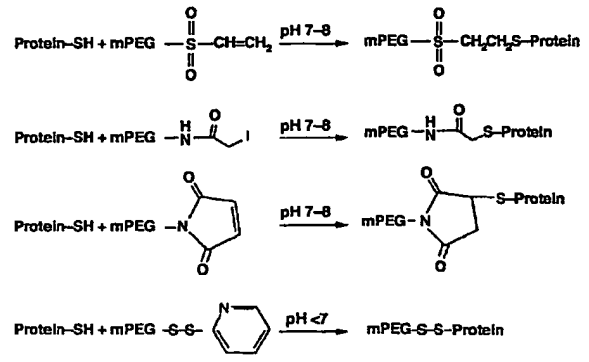
PEG itself has little toxicity when administered orally, intravenously, or topically.[1] PEG has a proven safety record; administration produces no significant toxicity, as shown in a number of studies[43] involving a variety of species. In addition, PEG has long been used as a vehicle or base for a large number of pharmaceutical preparations and foods. Despite this favorable history of use, one animal study[44] has suggested that PEG-linked proteins have the potential to induce renal tubular vacuolation. However, the effect was related to high doses of PEG/protein complex and was not associated with clinical effects or changes in functional markers.



(A) Site-directed medification of glycosylated proteins.



(B) Site-directed modification of N-terminus.



(C) Site-directed modification of free cysteine residues.

Figure 3. Site-directed pegylation of proteins.[39]

CLINICAL APPLICATIONS OF PEGYLATED PROTEINS

At present, two pegylated proteins are approved by the Food and Drug Administration, and a number of other pegylated agents are under investigation. PEG-adenosine deaminase (PEG-ADA, pegademase) is approved for the treatment of ADA deficiency in patients with severe combined immunodeficiency disease (SCID).[45] Prior to the availability of PEG-ADA, enzyme replacement was achieved by transfusion of irradiated red blood cells.[46] Pegademase is produced by coupling ADA to multiple strands of monomethoxypolyethylene glycol with a molecular weight of 5 kDa.[47] Coupling ADA with PEG decreases the immunogenicity of the protein and increases its half-life from a few minutes to approximately 24 hours.[48,49] In patients with SCID, treatment with pegademase has been shown to reverse the principal biochemical consequences of ADA deficiency and produce improvements in clinical symptoms.[47]

Pegylated asparaginase (pegaspargase) was the second pegylated product made available for use in the US and is approved for the treatment of acute lymphoblastic leukemia. Since leukemic cells are dependent on host cells to provide asparagine, the administration of asparaginase (which deaminates asparagine) produces a rapid depletion, thereby killing leukemic cells.[50] Although active, the native molecule is associated with a short half-life and high incidence of allergic reactions. Similar to PEG-ADA, pegylated asparaginase involves the covalent attachment of multiple units (5 kDa) of monomethoxypolyethylene glycol to the native compound. This produces a more stable molecule[51] with a greatly extended half-life in comparison to the unmodified molecule (357 vs. 20 h, respectively).[52] Pegylation of asparaginase also reduces the tendency of the enzyme to induce an immune response.

PEGYLATED THERAPEUTIC PROTEINS IN DEVELOPMENT

Preclinical studies have demonstrated that a number of other pegylated therapeutic proteins are associated with enhanced pharmacologic activity compared with the native molecule. For example, pegylation has been shown[53] to increase the plasma half-life of PEG-interleukin-6, resulting in augmentation of the thrombopoietic activity of the agent and a decrease in adverse effects. Pegylation of growth factors, such as growth hormone-releasing factor[54] and megakaryocyte growth and development factor,[55] have

been shown to enhance the biologic activity compared with the native compounds. Pegylation has also been used in combination with a vector-mediated blood–brain barrier drug delivery system to optimize the brain uptake of brain-derived neurotrophic factor.[56]

Interferon alfa has been the subject of extensive pegylation research. Attachment of PEGs of varying length to interferon alfa-2a has produced increases in the circulating half-life and increases in serum levels of antiviral activity.[57] The in vivo biologic activity of pegylated interferon alfa-2a has been shown to be directly correlated to the increasing mass of PEG.[41] Attachment of a 40 kDa branched-chain PEG to interferon alfa-2a is associated with sustained therapeutic interferon concentrations and a tenfold decrease in systemic clearance compared with standard interferon alfa-2a and a linear, 5 kDa peginterferon alfa.[58,59] Also, the branched, 40 kDa peginterferon alfa-2a is not associated with the wide fluctuations in serum interferon concentrations seen with standard interferon.[58] This may result in sustained pressure on the virus in diseases such as hepatitis C that require sustained drug concentrations to prevent the occurrence of viral drug resistance.[60] The hepatitis C virus has a short half-life and a high production rate (similar to HIV) and, since there is a dose-dependent effect of interferon on the clearance of viral RNA from the serum, standard interferon schedules may be associated with viral rebound during the days the patient is not receiving treatment.[60] The sustained therapeutic interferon concentrations allow for a more convenient dosage schedule (once a week vs. three times a week) and, therefore, allow for extended exposure to clinically effective concentrations of therapeutic interferon. In addition, pegylated interferon alfa-2a has been demonstrated to produce a similar drug metabolism profile on cytochrome P450 enzymes compared with that of unmodified interferon.[61]

Preliminary data suggest that pegylated interferon alfa-2a is clinically superior to standard interferon in patients with chronic hepatitis C. Pegylated interferon 180 µg once weekly produced sustained virologic responses in 36% of patients, compared with 5% of patients receiving standard interferon 3 MU three times weekly.[62] Branched, 40 kDa peginterferon alfa-2a has the potential to combine improved efficacy with a reduction in acute adverse effects. A product combining interferon alfa-2b with a single linear-chain PEG molecule is also undergoing clinical trials.[63] Previous experience suggests that optimization of the PEG moiety will result in optimized clinical features and that the differences in the pegylation process may show differences in clinical efficacy between these two pegylated interferons.

EFFECT OF PEGYLATION ON PHARMACOKINETIC PARAMETERS

Pegylated therapeutic proteins have pharmacologic and clinical properties unique to each molecule. Therefore, it is important to establish pharmacologic and clinical parameters that best define the efficacy of each molecule. For instance, the term half-life is a part of the vernacular when discussing drug delivery; however, because each pegylated therapeutic protein has unique intrinsic pharmacology and biologic activity, it becomes necessary to use more accurate terminology, such as absorption half-life and elimination half-life.

For example, pegylation clearly alters the absorption, distribution, and clearance of interferon in a size-dependent manner.[58,59] Pegylation appears to increase the elimination half-life of all interferons to a similar extent compared with standard interferon. However, both the 5 kDa, linear peginterferon alfa-2a,[59] and pegylated interferon alfa-2b[63] have absorption half-lives similar to that of standard interferon (2.3 h), whereas the 40 kDa, branched peginterferon alfa-2a[58] has an absorption half-life of 50 hours. The extended absorption half-life may account for the sustained maximal serum concentrations of the 40 kDa, branched peginterferon alfa-2a (>150 h) compared with those of pegylated interferon alfa-2b (48–72 h), the 5 kDa, linear peginterferon alfa-2a (<24 h), and standard interferon (<24 h).[58,59,63] Clearly, a generic discussion of half-life would not suffice to describe the distinct pharmacologic differences between these pegylated interferons.

Summary

To improve the delivery of injectable pharmaceutical agents, a number of novel drug delivery mechanisms have been developed. Continuous-release injectable formulations can provide more consistent pharmacodynamic effects, minimize adverse effects, and increase patient compliance. The success of these continuous-release mechanisms has been variable. Liposomal delivery has been pursued to alter tissue distribution of the unbound drug, resulting in an improvement in efficacy and a reduction in toxicity. However, liposomal formulations are associated with a number of limitations and, in general, have not met expectations. It is hoped that newer liposomal formulations (e.g., PEG-liposomes, immunoliposomes) will overcome these limitations.

While continuous-release and liposomal systems alter the formulation of the drug, pegylation alters the drug molecule. This technology may provide the best approach for enhancing the delivery of injectable agents by producing improvements in the pharmacokinetic and pharmacodynamic properties of molecules. Pegylation appears to be particularly useful in modifying proteins and has the potential to overcome many of the problems associated with the use of therapeutic proteins and peptides. Recent advances in pegylation technology have further enhanced the application of this technology. The key to optimal clinical benefit derived from the pegylation of therapeutic proteins is the careful optimization of the PEG moiety and the pegylation process. Each protein is different and requires different optimization chemistries on a protein-by-protein basis to acquire the maximum therapeutic effectiveness. In addition, a more precise language is needed to distinguish

between the pharmacologic properties of each pegylated therapeutic protein.

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EXTRACTO

OBJETIVO: Revisar el desarrollo reciente de nuevos mecanismos de liberación de fármacos inyectables y esbozar las ventajas y desventajas de cada uno.

FUENTES DE INFORMACIÓN: Se realizó una búsqueda en MEDLINE utilizando los términos polietileno glicol, liposomas, polímeros, ácido poliláctico, y liberación controlada. Referencias adicionales fueron identificadas examinando las bibliografías.

SELECCIÓN DE FUENTES DE INFORMACIÓN Y MÉTODO DE EXTRACCIÓN DE INFORMACIÓN: Todos los artículos fueron considerados para inclusión. Los extractos fueron incluídos solamente si se juzgó que los mismos añadían información crítica que de otra manera no estaba disponible en la literatura médica.

SÍNTESIS: Se han desarrollado un número de sistemas que alteran la liberación de fármacos inyectables con la intención de mejorar las propiedades farmacodinámicas y farmacocinéticas de agentes terapéuticos. Nuevos sistemas de liberación de fármacos pueden ser producidos a través de un cambio en formulación (e.j. productos de liberación continua o liposomas) o a través de una adición a la molécula del fármaco ("pegilación"). Posibles ventajas de los nuevos mecanismos de liberación incluyen duración de actividad farmacológica aumentada o prolongada, una disminución en efectos adversos, y un incremento en el cumplimiento de pacientes y calidad de vida. Sistemas de liberación continua inyectables liberan fármacos de manera controlada y predeterminada, y son apropiados especialmente para fármacos donde es importante evitar grandes fluctuaciones en las concentraciones en plasma. El encapsular un fármaco dentro de un liposoma puede producir una vida media prolongada y un cambio en distribución hacia tejidos con permeabilidad capilar aumentada (ej. tumores, tejido infectado). Pegilación provee un método para la modificación de proteínas terapéuticas con el propósito de minimizar muchas de las limitaciones que están asociadas con estos agentes (ej. estabilidad pobre, vida media corta, inmunogenicidad).

CONCLUSIONES: Pegilación de proteínas terapéuticas es un proceso establecido, con nuevas aplicaciones. Sin embargo, no todas las proteínas pegiladas son iguales, y cada una requerirá que se alcance el grado óptimo, en base a proteína por proteína, para obtener el beneficio clínico máximo. El lenguaje requerido para describir cada proteína terapéutica pegilada debe ser más preciso para poder distinguir con exactitud las propiedades farmacológicas que diferencian cada proteína.

Brenda R Morand

RÉSUMÉ

OBJECTIF: Réviser les développements récents dans les approches novatrices concernant les nouveaux systèmes de distribution des médicaments.

SOURCES DES DONNÉES: Une recherche MEDLINE utilisant les termes polyéthylène glycol, liposomes, polymères, acide polyacétique, et préparations à libération contrôlée a été efectuée.

SÉLECTION DES ÉTUDES: Tous les articles ont été évalués. Les résumés ont été inclus seulement s'ils apportaient de l'information complémentaire.

RÉSUMÉ: Un nombre important de systèmes qui modifient la distribution des médicaments par voie parentérale ont été développés pour améliorer les propriétés pharmacodynamiques et pharmacocinétiques des médicaments. La mise au point de ces nouveaux systèmes, dans le but de modifier la libération des principes actifs ou d'améliorer le passage des barrières physiologiques, utilise par exemple des liposomes ou le processus de pégilation. Les avantages de ces nouveaux systèmes comprennent: une augmentation ou une durée prolongée de l'activité pharmacologique, une diminution des effets indésirables, et une augmentation de la fidélité au traitement et de la qualité de vie du patient. Ces systèmes utilisent une libération des principes actifs dans un environnement contrôlé ce qui permet d'éviter des fluctuations dans les concentrations plasmatiques. En encapsulant un principe actif dans un liposome, on peut augmenter sa demi-vie et améliorer son passage dans la circulation sanguine. La pégilation s'avère une méthode intéressante pour la libération de protéines thérapeutiques et permet d'atténuer les problèmes associés soit la stabilité et la demi-vie du principe actif.

CONCLUSIONS: La pégilation des protéines thérapeutiques représente un processus déjà établi. Cependant, il existe des différences entre les protéines pégilasées; chacune des protéines utilisées dans ce processus doit être optimisée pour bénéficier d'une efficacité clinique maximale.

Louise Mallet

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Modulation of the Pharmacokinetics and Pharmacodynamics of Proteins by Polyethylene Glycol Conjugation

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PDF version for printing

Introduction

With the rapid advances in the field of biotechnology during the last decade, many peptides and proteins have been produced and evaluated for therapy of various diseases, including cancer. However, rapid clearance and the possibility of immunogenicity after the in vivo administration of these biotechnology-driven products have impeded their marketing. To circumvent these problems, synthetic and natural polymers such as polyethylene glycol (PEG) and dextrans, respectively, have been covalently attached to proteins, and some of these protein-polymer conjugates have shown promising therapeutic results. The conjugation of proteins with polymers usually causes a reduction in the recognition of the protein by the immune system, resulting in a decrease in protein clearance and immunogenicity. Most of the protein-polymer conjugates retain the pharmacologic activity of the protein, although to a lesser extent than the native protein. Additionally, in most of the examples in the literature, a significant increase in the plasma half life of the protein more than compensates for any reduction in the pharmacologic effects of the polymer-protein conjugates. Therefore, polymer conjugation in most cases would result in a net increase in the pharmacologic activity of the protein.

The intent of this article is to review the pharmacokinetics and pharmacodynamics of proteins conjugated to PEG which is one of the most widely used synthetic polymers for protein conjugation.

Physiochemical Properties

protein. In most cases, the activating agent acts as a linker between PEG and the protein, and several PEG molecules may be attached to one molecule of protein as depicted in Figure 1. Therefore, pharmacokinetics and pharmacodynamics of the PEG-protein conjugates are dependent on the MW of the PEG used for conjugation, the number of PEG molecules per each molecule of protein, and the nature of the bond between the protein and the linker. Interested readers are referred to a comprehensive review of the PEG-protein coupling methods by Deluged et al. (1).

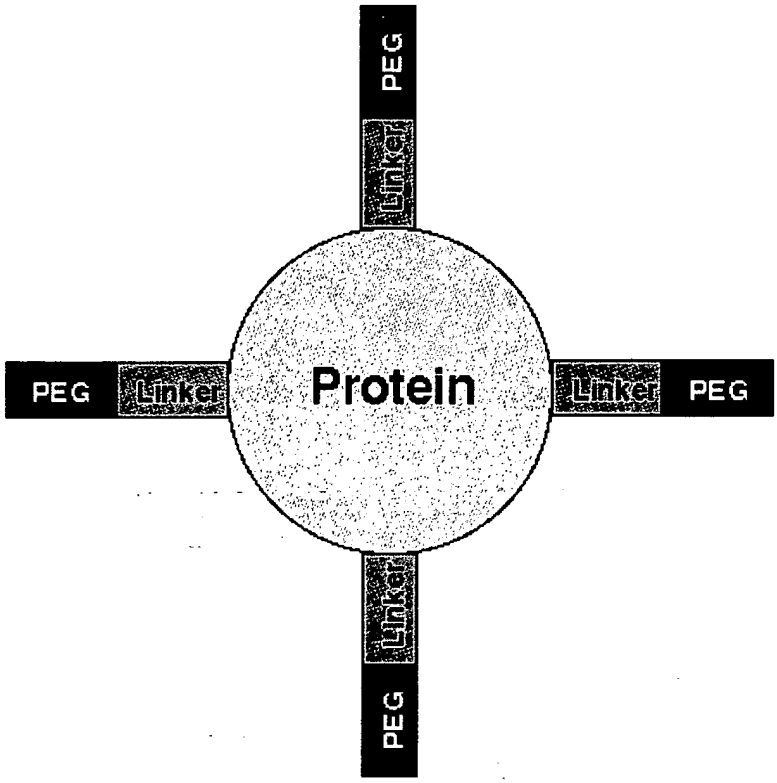

Figure 1. Schematic presentation of a protein-PEG conjugate. The number of PEG molecules per each protein molecule varies for different conjugates.

In Vivo Disposition of PEG Backbone

It is believed that the kinetics of proteins attached to polymers are substantially affected by the kinetics of the polymer itself. Therefore, before reviewing specific PEG-protein conjugates, an analysis of the plasma kinetics and tissue distribution of PEGs is necessary.

The plasma kinetics of PEGs are reported (2, 3) to be dependent on both the MW of the polymer and the site of injection. Yamaoka et al. (2) investigated the disposition of radiolabeled PEGs with MWs of 6 kD (PEG-6), 20 kD (PEG-20), 50 kD (PEG-50), and 170 kD (PEG-170) after iv administration to mice. Similar to other polymers such as dextrans (4, 5), the plasma concentrations (Fig. 2) and area under the plasma concentration-time curves (AUCs) (Table 1) of higher MW PEGs were substantially greater than those of the lower MW polymers. Additionally, the half life of the polymers progressively increased as the MW increased from 6 kD to 170 kD (Table 1); the relationship between the half and the MW of PEGs is sigmoidal (Figure 3), which appears to be one of the characteristics of the kinetics of macromolecules.

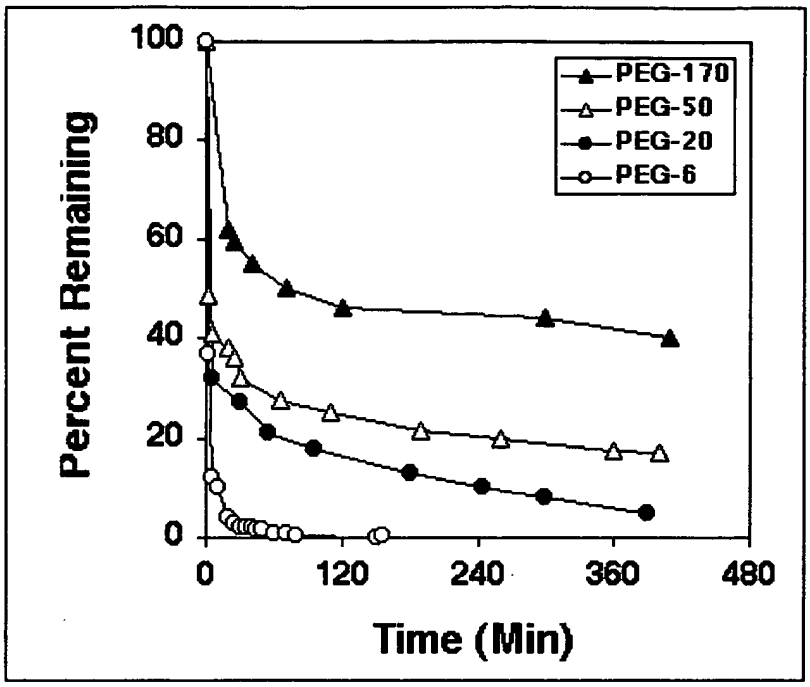

Figure 2. Blood radioactivity-time courses after iv administration of PEG with different molecular weights. Key: (s) PEG-170; (Δ) PEG-50; (l) PEG-20; (m) PEG-6. From Ref. (2).

Table 1. Mean ± SD of AUC and terminal half life of PEGs with different MWs after iv administration to mice[a]

Parameter	PEG-6	PEG-20	PEG-50	PEG-170
AUC,	6.17	110	600	1110
%dose.hr/mL	± 2.18	± 7.17	± 11.9	± 27.0
$t_{1/2}$, min	17.6	169	987	1390
	± 5.90	± 20.0	± 79.0	± 57.0

[a] *Source: Reference (2)*

With regard to the site of injection, PEG-50 is retained at the injection site longer than PEG-6 after im and sc injections (3), suggesting that the absorption of PEG from im and sc sites is MW dependent. However, after the ip administration, the injection site disappearance profiles of both MWs were very similar (3).

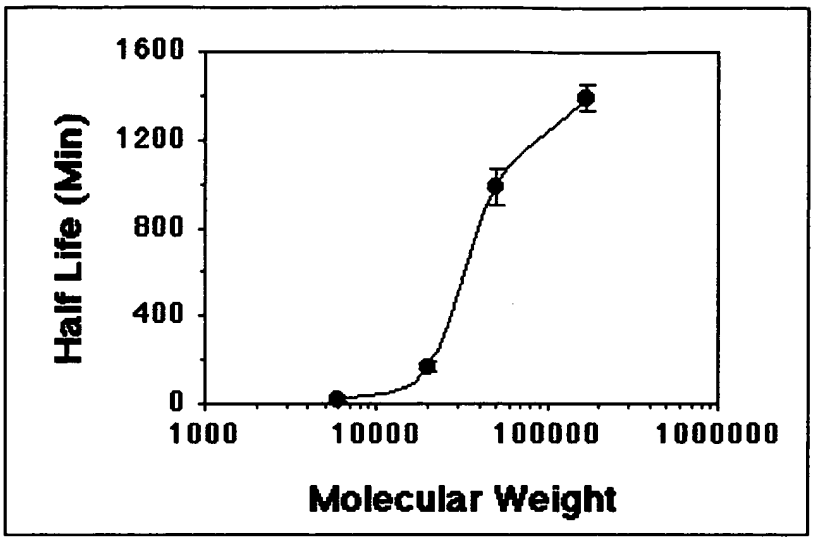

Figure 3. Relationship between the plasma half life of PEG and its molecular weight. From data presented in Table 1, Ref. (2).

The differences among the plasma concentration-time courses of PEGs with different MWs (Fig. 2) are mostly due to the size of these PEGs in relation to the pore sizes of the vascular beds in kidneys. Chang et al. (6) reported that, in rats, renal elimination of another linear polymer, neutral dextrans, with a MW of ~ 10 kD occurred without any molecular restriction. However, the renal clearance of dextrans of larger MWs progressively decreased and approached zero at a MW of ~40 kD. This is in agreement with a study (2) in mice using radiolabeled PEG, demonstrating a sigmoidal relationship between the renal clearance and the log MW of PEGs. This type of sigmoidal relationship (2) agrees well with the theoretical models of renal excretion of macromolecules based on the pore sizes of the glomerular capillary wall.

The relatively limited information on the metabolism of PEG in the body (7, 8) indicates that PEG undergoes cytochrome P-450 oxidation, resulting in the formation of ketone, ester, and aldehyde groups (8). Additionally, smaller MW PEGs are excreted into bile (7).

In terms of tissue distribution, it appears that PEGs with MWs between 6 kD to 170 kD distribute insignificantly to tissues such as heart, lung, liver, spleen, kidney, and thyroid gland (2). However, the distribution of PEGs to gastrointestinal tract and feces is relatively substantial (2). Additionally, no clear MW dependency is observed for the accumulation of PEG in tissues (2).

PEG-Protein Conjugates

During the last three decades, PEG has been investigated extensively for delivery of various proteins via parenteral routes. Some examples are listed below.

Anticancer Agents

Generally, polymers have been most widely used for the delivery of both traditional (small molecule) drugs and proteins/enzymes in the treatment of cancer. However, PEGs have been specifically investigated for the delivery of anticancer proteins/enzymes as discussed below.

Antibodies: One of the major problems for the use of xenogenic monoclonal and polyclonal antibodies for the treatment of tumors is their immunogenicity which results in a rapid removal of the antibodies from the body and the possibility of allergic reactions after multiple administration. Kitamura et al. (9) conjugated the F (ab')2 fraction of the murine monoclonal antibody A7 to PEG 5 kD and studied the tumor accumulation and the kinetics of the conjugate in mice. The conjugate had a longer plasma half life and higher tumor accumulation, compared with the free F (ab')2 fraction. However, the tumor: blood ratio of the free F(ab')2 fraction was higher than that for the conjugate (9).

Takashina et al. (10) studied the pharmacokinetics and dynamics of conjugates of monoclonal antibody A7 to PEG 5 kD and dextran 70 kD. In vitro studies showed that the conjugates retained the antigen binding activity of the antibody. Additionally, after the iv administration of the conjugated and free antibody, the PEG conjugate had a plasma half life twice of that for the free antibody (10). On the other hand, the dextran conjugate showed higher clearance and shorter half life, compared with the free and PEG conjugated antibody. Additionally, the tumor accumulation of dextran-antibody conjugate was less than those for the free and PEG conjugated antibody. This study (10) suggests that the kinetics of polymer-monoclonal antibody A7 are significantly dependent on the structure of the polymer.

Arginase: A PEG 5 kD conjugate of arginase retained 65% of the activity of the enzyme and prolonged its plasma half life in mice after multiple dose therapy (11); 30 days after the start of the treatment, the half life of the native enzyme was 1 hr, while the half life of the conjugate was 12 hr. In terms of effects, the conjugate increased the survival time in mice with Taper liver tumor. However, the free enzyme did not show any improvement in the survival time (12). With regard to the effects of the enzyme against L5178Y mouse leukemia cells, whereas the conjugate was more effective than the native enzyme in vitro, neither was able to stop the growth of tumor in vivo (12).

Asparaginase: Asparaginase, isolated from Escherichia Coli and Erwinia Carotovora, metabolizes asparagine, a necessary nutrient for sensitive tumors. However, after multiple injection of the enzyme, antibodies raised against the enzyme would quickly remove the enzyme from the circulation, and also significant immunogenicity may be observed. Several studies (13-19) have documented the usefulness of a conjugate of asparaginase with PEG for the treatment of various cancers in both humans and animals. Ho and his colleagues (15, 17) showed that the conjugate would alter the pharmacokinetics of the enzyme drastically in both humans and rabbits. In humans (15), conjugation resulted in an increase in the plasma half life from 20 hr (for native enzyme) to 357 hr (for the conjugate). In rabbits (17), the half life values of the free and conjugated asparaginase were 20 and 144 hr, respectively. The increase in the plasma half lives in both species was due to a significant decrease in the clearance of the enzyme (15, 17). The alterations in the kinetics of the enzyme by PEG conjugation also resulted in significant improvements in the toxicity and efficacy profile of the enzyme after in vivo administrations to animals (14, 16, 18) and humans (13, 19). A conjugate of asparaginase and PEG (pegaspargase) was marketed (Oncaspar®) in 1994 for the treatment of acute lymphoblastic leukemia (ALL) in patients who are hypersensitive to native forms of L-asparaginase. Oncaspar® is marketed by Rhône-Poulenc Rorer Pharmaceuticals, Inc. in the U.S. and Canada.

Methioninase: It is known that all the tumor cells have elevated requirement for

methionine. Therefore, methioninase may be used in cancer therapy. However, the recombinant enzyme, obtained from bacteria, has a short plasma half life and may be immunogenic upon multiple dose administration. Very recently, Tan et al. (20) demonstrated the potential of a conjugate of methioninase and PEG 5 kD in cancer therapy. In vitro tests demonstrated that the conjugate retained 70% activity of the enzyme. Additionally, in rats, the plasma half life of the enzyme was increased by a factor of 2 when it was conjugated to PEG 5 kD (20). Further, the effects of the conjugate lasted for 8 hr, as opposed to 2 hr for the free enzyme. In vitro studies in human lung and kidney cancer cells showed identical IC50 values for the conjugated and free methioninase, demonstrating the effectiveness of the enzyme in the conjugated from. Also, after the injection of the conjugate to tumor-bearing mice, the tumor: blood enzyme ratio was higher for the conjugate (1:6), compared with the free enzyme (1:10) administration (20). More studies are needed to confirm these promising findings.

Enzyme Replacement

Adenosine Deaminase: A deficiency of the enzyme adenosine deaminase (ADA) results in combined immunodeficiency disease (CID). For several years, conjugates of PEG and ADA have been used successfully for enzyme replacement in the treatment of CID in children (21-23). A conjugate of PEG and ADA, which is also named pegademase, was marketed (Adagen®) by Enzon, Inc. (Piscataway, NJ) in the US in 1990. The outcome of therapy with the conjugate appears to be better than red blood cell transfusion (23), which is another treatment for ADA deficiency. Studies (21-23) have shown that weekly intramuscular injections of the conjugate of PEG with bovine ADA would reverse the symptoms of ADA deficiency in most cases without substantial toxicity or hypersensitivity. The conjugate appears to have a very long half life of 48-72 hr in children (21). From a historic perspective, the PEG-ADA conjugate served as one of the earliest examples of polymer conjugates marketed in the US and prompted more research interest in this area.

Uricase: Uricase is an enzyme which converts uric acid to allantoin and is lacking in humans. When the enzyme is administered to humans, it causes a significant reduction in the plasma and urine levels of uric acid. Therefore, it can be effective in the treatment of gout and other diseases related to high levels of urate. However, after multiple administration, the antibodies against this enzyme would deactivate it very rapidly. Several conjugates of uricase with PEGs (24-28) have been investigated to overcome this problem. Yasuda et al. (28) reported that conjugation of uricase with PEG resulted in a decrease in antibody production and reactivity towards uricase. When administered intravenously to rats, the enzymatic activity half life of the PEG conjugate (~ 7 hr) was almost 10 times of that for the parent enzyme (0.6 hr) (28).

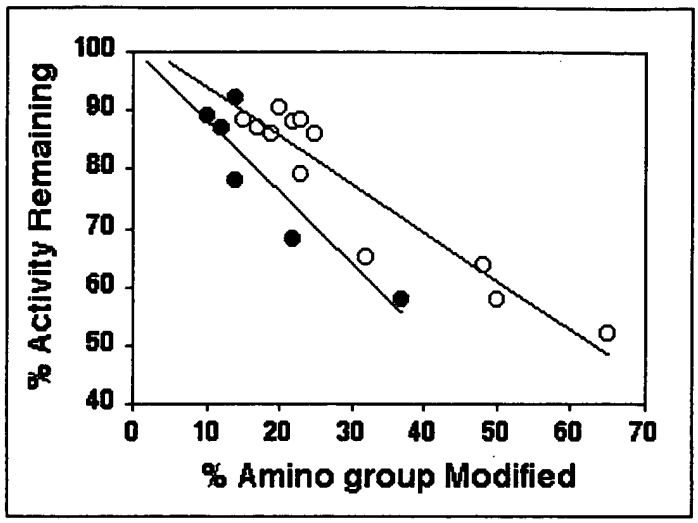

Figure 4. Relationship between the percentage of amino groups of uricase modified with dextran 10 kD (m) or PEG 10 kD (l) and the percentage of remaining enzymatic activity of uricase. From Ref. (28).

The enzymatic activity of the polymer conjugated uricase is shown to be dependent on the degree of modification of the amino groups of the enzyme during the conjugation process (28). An increase in the modification would result in a decrease in the enzymatic activity of uricase for both dextran and PEG conjugates (Fig. 4). However, the decrease in the activity is more pronounced for the PEG conjugate, compared with dextran conjugation (Figure 4) (28).

Antioxidant Enzymes

Catalase: Similar to superoxide dismutase (SOD), catalse is an antioxidant enzyme, and several studies (29-32) have investigated the effects of PEG conjugates of SOD and catalse on the same animal model. The PEG-catalse conjugate was first prepared by Abuchowski et al. (33) using both PEG 1900 and 5 kD. These investigators (33) demonstrated that both conjugates retained significant (>90%) activity of the enzyme and were resistant to digestion by trypsin, chymotrypsin, and protease. Further, the half life of the conjugates was long even after their repeated administration to mice (33). A later study (34) using subcutaneous osmotic pumps delivering the conjugate showed the conjugate's beneficial effects in a rat model of lung injury due to asbestosis. Despite promising effects of the PEG-catalase conjugate, recent work in this area has concentrated more on a conjugate of SOD and PEG described below.

Superoxide Dismutase (SOD): Among the conjugates of PEG, SOD is the most widely studied. Superoxide dismutase is an antioxidant enzyme which eliminates superoxide anion, reducing tissue injury. After its iv administration in animals, the plasma half life of the enzyme is very short (5-10 min). Several investigators have reported the effects of conjugation of SOD with PEG on the pharmacokinetics and dynamics of the enzyme, some of which are summarized in Table 2 (29-32, 35-47). Although some of these studies have compared the effects of PEG-SOD with those of the free enzyme, most of the studies have concentrated on the effects of PEG-SOD without a comparison with the free SOD (Table 2). There is little doubt that conjugation of SOD with PEG increases its plasma half life (35) and reduces its immunogenicity (29, 35). However, conflicting reports (30-32, 36-40, 43, 45-47) exist with regard to the effects of PEG-SOD in various animal models of injury. Additionally, the results of clinical trials (42, 44) with PEG-SOD have not been

unequivocal.

Table 2. Some of studies on the conjugates of PEG and SOD.

Type of Study	Comments	Reference
In vitro and in vivo kinetics and dynamics in rats	PEG 5 kD conjugate retained 51% enzyme activity; plasma half life of conjugate was longer than the native SOD after repeated dosing; anti-inflammatory effect of the conjugate was higher than SOD.	(35)
In vivo immunogenicity in mice	Decreased immunogenicity; antibody titer to the conjugate was 0.03%-0.07% of that observed with SOD.	(29)
In vivo effects in endotoxemia in pigs	No beneficial effects	(30)
In vivo effects in a dog model of ischemia/ reperfusion	Conflicting results: both no effect (37) or a reduction (36) in heart injury associated with reperfusion have been reported.	(36, 37)
In vivo effects in a rat model of brain ischemia	Administration of PEG-SOD before induction of focal cerebral ischemia resulted in a reduction in brain injury.	(31)
In vivo effects in a rabbit model of ischemia/ reperfusion	No effect in heart injury associated with reperfusion.	(38)
In vivo distribution into brain of piglets	IV injection of PEG-SOD did not increase the enzyme level in the brain in control piglets and in animals subjected to global ischemia/reperfusion.	(39)
In vivo effects in hemorrhagic shock in rats	Administration of a PEG-SOD conjugate to a rat model increased survival from 25% to 67%.	(40)
In vivo brain distribution in rats	The concentrations of PEG-SOD in the brain and CSF of normal rats were low; brain and CSF concentrations were higher after hypertensive brain injury	(41)
In vivo effects in piglets with hypoxic brain injury	Administration of PEG-SOD 5 min after reoxygenation did not have any positive effects.	(32)
Phase II clinical trial study in severe head injury	Improved outcome at 3 and 6 months after the treatment with PEG-SOD (10,000 U/kg), compared with placebo.	(42)
In vivo study in rats with oxygen toxicity	Insufflation of PEG-SOD increased survival time, in comparison with both placebo and free SOD.	(43)
Clinical trial in severe head injury	Percent of patients in a vegetative state or dead at 3 and 6 months postinjury was lower after the conjugate, compared with placebo.	(44)
In vivo effect in a rat model of ischemic renal failure	The PEG-SOD was more effective than an equivalent dose of free SOD.	(45)
In vivo effects in a rat model of warm renal ischemia	PEG-SOD conjugates were more protective, compared with free SOD.	(46)
In vivo effects in a rat model of ischemia/reperfusion	SOD conjugated to PEG showed a superior effect over that conjugated to polyacryloylmorpholine.	(47)

Thrombolytic Agents

Streptokinase: Rajagopalan et al. (48) conjugated streptokinase to PEG 2 kD, 4

kD, and 5 kD, and investigated the thrombolytic activity and antigenicity of the conjugates. In vitro studies demonstrated comparable activity for the conjugates and the free enzyme. However, the binding of the conjugates to antibodies against streptokinase was reduced by 95% (48). In vivo studies in mice (48, 49) revealed low clearance of the conjugates attached to plasmin, resulting in a half life of 200 min for the conjugate, compared to a half life of 15 min for streptokinase itself. These studies (48, 49) demonstrate that PEG conjugation of streptokinase retains the activity of the enzyme, prolongs its plasma circulation by blocking plasmin degradation, and reduces the antigenicity of the enzyme.

Urokinase: In dogs, a conjugate of urokinase, a thrombolytic agent, with PEG 5 kD was shown (50) to have longer activity and more activation of fibrinolysis, compared with the native enzyme. Also, a polypropylene glycol-PEG conjugate of urokinase showed a decreased activity on plasminogen and had a longer plasma half life in rabbits, compared with the native enzyme (51). Later (52), it was shown that this conjugate blocked autolysis of the enzyme at 37^0C. Unfortunately, these early positive results have not been followed by more extensive in vivo studies.

Oxygen Carriers

Hemoglobin: Several studies have examined the feasibility of the conjugation of hemoglobin to PEG for use as a blood substitute. Hemoglobin binds to oxygen and can be used as an oxygen carrier. However, because of its rapid elimination, the plasma half life of the protein is very short. Additionally, the affinity of hemoglobin to oxygen is too high for release of oxygen in the tissues. A conjugate of PEG with pyridoxylated hemoglobin has been shown (53, 54) to have longer plasma half life and better therapeutic effects in rats, compared with the free hemoglobin. The benefits of PEG-hemoglobin conjugates as a blood substitute have been shown in several animal models, including a hemorrhagic hypotension pig model (55) and in partial exchange transfusion and top-loaded rat models (56). Additionally, a PEG-hemoglobin conjugate has been used (57) for an increase in the sensitivity of tumors to radiation by increasing oxygen delivery to the tumor. These studies point to the potential of hemoglobin conjugated to PEG for manipulation of the oxygen levels in normal and malignant tissues.

Cytokines and Hematopoietic Growth Factors

Interleukin-2 (IL-2): Both animal and clinical studies have been conducted using PEG conjugates of IL-2. Earlier studies in animals (58, 59) and humans (60) showed that PEG conjugation would increase stability, decrease clearance, and increase plasma half life (> 20 fold) of IL-2. Further, these studies (58-61) suggested promising effects for the PEG-IL-2 conjugate in the treatment of various cancers. However, more recent data (62-64), mostly in patients, have failed to clearly demonstrate an advantage for PEG-IL-2, compared with free IL-2, in terms of therapeutic or toxic end points for the treatment of cancer. On the other hand, it appears that recent interest in the PEG-IL-2 conjugate revolves around its potential beneficial effects in patients with human immunodeficiency virus (65-69). Recent studies (65-69) in patients with HIV show that low dose PEG-IL-2, alone or in combination with zidovudine, would increase the immune response by increasing the number of CD4 T cells without significant toxicity. Additional clinical studies, comparing free and PEG conjugated IL-2 will shed more light on these exciting results.

Recombinant human granulocyte colony-stimulating factor (rhG-CSF): This is a 156 amino acid glycoprotein which is produced by Escherichia Coli and increases production and phagocytic and cytotoxic activities of neutrophils (70). The plasma half life of rhG-CSF is short (3.5 hr) (70), requiring daily injections to sustain the neutrophil levels in situations like cancer chemotherapy. In 1991, Tanaka et al. (71) reported that a conjugate of rhG-CSF with PEG increased the plasma half life of the growth factor from 1.8 hr (native factor) to 7 hr (conjugated factor) in mice. The increase in half life was associated with an increase in both the intensity and duration of the effect of the drug on the neutrophil count (71). These results were later (72) confirmed in mice made neutropenic by the administration of anticancer agents cyclophosphamide and fluorouracil. Recent studies (73-75) demonstrated that the in vivo activity of the conjugate is dependent on both the MW of PEG (73, 74) and the total number of PEG units attached to rhG-CSF (73, 75); there was a positive relationship between the total mass of the conjugate and the intensity and duration of the effect of rhG-CSF. Future studies should be conducted to determine whether these positive results in animals can be extended to humans.

Recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF): This is a 127 amino acid glycoprotein produced in yeast which acts similar to rhG-CSF to increase neutrophils, with a broader action on monocytes, macrophages, and eosinophils (70). Similar to rhG-CSF, the plasma half life of rhGM-CSF is short (2-3 hr) (70), requiring daily injections to sustain the neutrophil levels in patients undergoing bone marrow transplantation or intensive chemotherapy. Compared with rhG-CSF, the studies on the conjugates of PEG with rhGM-CSF are scarce (76, 77). The limited information indicates that similar to rhG-CSF, PEG conjugation increases the plasma half life (76) and some biological activities of rhGM-CSF (77).

Other Proteins

Table 3 lists the use of PEGs for delivery of some other therapeutic agents (78-86) which are not discussed in detail in this review. These studies (Table 3) show that polymer conjugation could result in altered pharmacokinetics, decreased affinity of the conjugate to bind to the protein receptor, and/or a decrease in antigenicity of proteins.

Concluding Remarks

The examples provided in this review clearly point to the potential advantages of polyethylene glycols for parenteral delivery of proteins. Despite significant promise of protein therapeutics in cell culture and other in vitro studies, optimal delivery of these agents in humans is very challenging. This is mostly because of relatively high clearance and short plasma half life of these agents, especially after multiple administration which results in activation of the immune system and faster elimination of the proteins. The available studies on the use of PEG for delivery of proteins indicate that these polymers will continue to have a significant role in the delivery of proteins in the future.

Table 3. Additional studies on the conjugates of PEGs with proteins

Drug/Protein	Description
Antigen E	A preliminary study in man showed that a 5 kD conjugate may be useful for the immunotherapy of ragweed hay fever (78).
Batroxobin	A 10 kD conjugate retained the activity of the enzyme while losing its ability to bind to anti-batroxobin antibodies in dogs (79).

Bilirubin oxidase	In a rat model of jaundice, the conjugate reduced the blood and liver levels of bilirubin, but, did not improve the liver function tests (80).
Honeybee Venom	In a clinical study, a 5.7 kD conjugate showed lower systemic reactions during immunotherapy and less efficacy against honeybee sting (81).
Interferon-alpha	In humans, the half life of the conjugate was twice as long as that of free protein; however, this did not result in a substantial reduction in the frequency of the protein administration (82).
Interferon-gamma	A 5 kD conjugate had activity similar to that of free protein but with a reduced binding affinity; the plasma half life of the conjugate was significantly longer than that of free protein in rats (83).
Interleukin-6	A 12 kD conjugate showed significantly higher thrombopoietic effects (increase in the platelet counts), compared with free IL-6 in mice (84)
Tissue Plasminogen Activator	In mice, the half life of radioactivity after the injection of the radiolabeled conjugate with 5 kD and 20 kD was long; however, the effect disappeared much faster (85).
Trypsin	A 5 kD conjugate was resistant to anti-trypsin antibody precipitation and retained some of the activities of trypsin to varying degrees (86).

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10/081,309	02/21/2002	Michael Brandt	20859	3846

151 7590 09/02/2005

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EXAMINER	
CHANDRA, GYAN	
ART UNIT	**PAPER NUMBER**
1646	

DATE MAILED: 09/02/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

RESPONSE TO FINAL REJECTION & NOTICE OF APPEAL DUE: December 2 2005

STATUTORY PERIOD EXPIRES: March 2 2006

PATENT DEPARTMENT
SEP - 8 2005

PTO-90C (Rev. 10/03)

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Office Action Summary	Application No.	Applicant(s)
	10/081,309	BRANDT ET AL.
	Examiner	Art Unit
	Gyan Chandra	1646

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *29 June 2005*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-15* is/are pending in the application.

4a) Of the above claim(s) *3,7 and 9-11* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,2,4-6,8 and 12-15* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *21 February 2002* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *6/29/05*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Status of Application, Amendments, And/Or Claims

Applicants have confirmed Restriction/Election made on December 22, 2004. Therefore, the Restriction/Election is made final.

The text of those sections of Title 35, U.S. Code, not included in this action can be found in a prior office action.

Information Disclosure Statement

The information disclosure statement filed 2/21/2002 fails to comply with 37 CFR 1.98(a)(1), which requires the following: (1) a list of all patents, publications, applications, or other information submitted for consideration by the Office; (2) U.S. patents and U.S. patent application publications listed in a section separately from citations of other documents; (3) the application number of the application in which the information disclosure statement is being submitted on each page of the list; (4) a column that provides a blank space next to each document to be considered, for the examiner's initials; and (5) a heading that clearly indicates that the list is an information disclosure statement. The information disclosure statement has been placed in the application file, but the information referred to therein has not been considered.

Rejections/Maintained

Claim Rejections - 35 USC § 103(a)

The rejection of claims 1-2, 4-6, 8, and 12-13 under 35 U.S.C. 103(a) as being unpatentable over Namiki et al in view of Date et al and Gaertner et al, is maintained for reasons of record in the previous Office Action.

Applicants argue that Nimiki et al only teaches the modification of HGF by attaching PEG(s) at the N-terminus amino acids to improve the clearance in vivo pharmacokinetics of HGF but they do not teach (a) the modification of NK4, (b) adding a monoethoxy linear PEG, and they do not teach adding a PEG of molecular weight 20-40kDa. Applicants' argument has been fully considered but is not found to be persuasive because Nimiki et al teach that the modification of HGF by attaching monoethoxy linear and branched PEG(s) at the N-terminus amino acids to improve the clearance and in vivo pharmacokinetics of HGF and Date et al teach that HGF comprises a four-kringle-containing (NK4). They teach that the NK4 is a 59 kDa protein. Further, Gaertner et al teach attaching PEG at amino terminus of proteins and suggest that a PEG in size from 5 to 40 kDa should be attached to a protein for an improved bioavailability. The skill of art is high and it would have been prima facie obvious to the person of ordinary skill in the art at the time the invention was made to attach PEG molecules in the range of molecular weight 5-40 kDa (as suggested by Gaertner) to the N-terminus amino acid of NK4 in order to increase clearance and pharmacokinetics of the molecule.

In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986).

The rejection of claims 14-15 under 35 U.S.C. 103(a) as being unpatentable over Namiki et al in view of Date et al and Gaertner et al as applied to claims 1-2, 4-6, 8, and 12-13 above, and further in view of Veronese et.al, is maintained. Applicants argue that because the rejection of claims 1-2, 4-6, 8, and 12-13 under 35 U.S.C. 103(a) does not hold therefore, claims 14-15 are in condition for allowance. Applicants' argument has been fully considered but is not found to be persuasive for the reasons of records and as set forth supra.

Conclusion

No claim is allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Gyan Chandra whose telephone number is (571) 272-2922. The examiner can normally be reached on 9:00-5:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anthony Caputa can be reached on (571) 272-0829. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Gyan Chandra
AU 1646
25 August 2005

JANET L. ANDRES
SUPERVISORY PATENT EXAMINER

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use several sheets if necessary)

Complete if Known	
Application Number	10/081,309
Filing Date	Feb. 21, 2002
First Name Inventor	Michael Brandt
Group Art Unit	1644
Examiner Name	Chandra, G.
Attorney Docket Number	20859

Sheet 1 of 1

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	U.S. Patent Document Number	Kind Code[2] (if known)	Name of Patentee or Applicant of Cited Document	Date of Publication of Cited document MM-DD-YYYY
GC	A3	6,583,272		Hoffmann-La Roche Inc	06-24-2004
GC	A4	6,340,742		Roche Diagnostics GmbH	01-22-2002
GC	A5	2002/0115833		Burg, Joseph et al	08-22-2002

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Office[3]	Number[4]	Kind Code[5] (if known)	Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	T[6]
Examiner Signature		Date Considered			Gyan Chandra	8/26/05	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Unique citation designation number. [2] See attached Kinds of U.S. Patent Documents. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] Applicant is to place a check mark here if English language Translation or abstract is attached.

158804

AS/REPLY/RH

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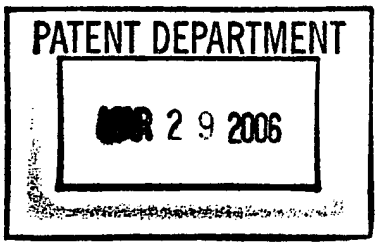
APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/081,309	02/21/2002	Michael Brandt	20859	3846

151 7590 03/24/2006

HOFFMANN-LA ROCHE INC.
PATENT LAW DEPARTMENT
340 KINGSLAND STREET
NUTLEY, NJ 07110



EXAMINER	
CHANDRA, GYAN	
ART UNIT	PAPER NUMBER
1646	

DATE MAILED: 03/24/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Appeal Brief rec'd Docket for May 6, 2006
bnk

PTO-90C (Rev. 10/03)

Copy Sent to
~~Department PLP~~
Persbeeg

Advisory Action Before the Filing of an Appeal Brief	**Application No.** 10/081,309	**Applicant(s)** BRANDT ET AL.	
	Examiner Gyan Chandra	**Art Unit** 1646	

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 05 January 2006 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:
   a) ☒ The period for reply expires 3 months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

   Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☒ The Notice of Appeal was filed on *06 March 2006*. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3 ☐ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   (a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);
   (b) ☐ They raise the issue of new matter (see NOTE below);
   (c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   (d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

   NOTE: _____. (See 37 CFR 1.116 and 41.33(a)).

4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): a) ☐ will not be entered, or b) ☒ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.

   The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: _____.
   Claim(s) objected to: _____.
   Claim(s) rejected: *1,2,4-6,8 and 12-15*.
   Claim(s) withdrawn from consideration: *3,7 and 9-11*.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing a good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

11. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: see continuation sheet.
12. ☒ Note the attached Information Disclosure Statement(s). (PTO/SB/08 or PTO-1449) Paper No(s). 1/5/2006
13. ☐ Other: _____.

Continuation of 11 does not place the application in condition for allowance because:

Claims 1-15 are pending. Claims 3, 7, and 9-11 are withdrawn.
Claims 1-2, 4-6, 8, and 12-15 are under examination.
The Information Disclosure Statement (IDS) filed on 1/5/06 replacing a blank sheet due to scanning error has been considered.

Applicant's Response to Final Rejection filed on 9/02/2005 is acknowledged. The rejection of claims 1-2, 4-6, 8, and 12-13 under 35 USC 103 (a), is maintained for the reasons of record in the previous office action mailed on 9/02/2005.
The claimed invention is drawn to a conjugate consisting of a NK4 molecule and a polyethylene glycol group having a molecular weight of about 20-40 kDa wherein polyethylene glycol group has: (i) the formula–CO-(CH2)X-(OCH2CH2)mOR, (ii) is monomethoxy polyethylene glycol and (iii) forms amide group with the amino groups of N-terminal NK4 fragment.

Applicant argues that Nimiki only teaches only modifying HGF via "monoethoxy linear PEG" and would not be applicable to other proteins, in particular if they comprise amino acids other than serine and threonine. Gaetner et al. Teach PEGylation of N-terminus amino acid through Oxime bond formation. Applicant points to Mehvar (2000) and Reddy (2000) references that the PEGylation of IL-8 and G-CSF causes impaired activity and that each protein requires different optimization chemistry.

Applicant's response have been fully considered but it is insufficient to overcome the rejection of claims 1-2, 4-6, 8, and 12-13 under 35 USC 103 (a), as set forth in the last Office action and further because the skill in the art of PEGylation of proteins at N-terminus of a protein is high. For example, Lu and Felix (see abstract: Int J Pept Protein Res 43: 127-138, 1994), Gonzalez et al. (US Patent No. 6, 133,426) teach PEGylation of protein and peptides at the N-terminus by the formation of a covalent bond with -NH2 of the N-terminus amino acid. It is also known in the art that some proteins may be subopitmally active when PEGylation is achieved at the N-terminus and therefore, site specific PEGylation is well practiced. Date et al disclose that within HGF is a four-kringle-containing (NK4) growth factors and the importance of NK4 in growth and invasiveness of carcinoma cells. Namiki et al teach that the modification of HGF by attaching monoethoxy linear and branched PEG(s) at the N-terminus amino acids improves the clearance and in vivo pharmacokinetics of HGF (page 2, line 49-57), as set forth in the previous Office Action. It would have been prima facie obvious to the person of ordinary skill in the art at the time the invention was made to attach PEG molecules to the N-terminus amino acid of NK4 in order to increase clearance, improve in vivo pharmacokinetics as taught by Namiki with a HGF protein, and to prepare a pharmaceutical composition comprising the PEGylated protein. Even if some pegylation were to decrease activity of the protein, it would have been obvious to experimentally determine which types of pegylation would result in a protein with improved characteristics.

The rejection of claims 14 and 15 under 35 U.S.C. 103(a) as being unpatentable over Namiki et al in view of Date et al and Gaertner et al as applied to claims 1-2, 4-6, 8, and 12-13 above, and further in view of and further in view of Veronese et.al. (US Patent 6,528,485 B1), is maintained for the reasons of record in the previous office action mailed on 9/02/2005.

Applicant argues that the Veronese reference is focused on HGRF only and that they do not address PEGlyation of any other protein, such as NK4.
Applicant's arguments have been fully considered but they are not found to be persuassive because the teacings of Date et al in combination with Namiki et al make PEGylation of NK4 obvious as set forth above. Veronese et al. teach making PEGylated proteins and purifying them to greater than 92% purity. Veronese et al teach that with high purity a PEGylated protein would result in a better bioavailability and pharmacokinetics in vivo.
In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See In re Keller, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); In re Merck & Co., 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986).

Eileen B. O'Hara

EILEEN B. O'HARA
PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 10/081,309	Applicant(s)/Patent Under Reexamination BRANDT ET AL.	
	Examiner Gyan Chandra	Art Unit 1646	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,133,426	10-2000	Gonzalez et al.	530/388.23
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Lu and Felix, Int J. Pept. Protein Res. (abstract), 1994.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use several sheets if necessary)

Complete if Known	
Application Number	20859
Filing Date	February 21, 2002 with Express Mail Label No. EL912162005 US
First Name Inventor	Brandt, M. et al.

Sheet 1 of 1

U.S. PATENT DOCUMENTS

Examiner Initials'	Cite No.[1]	U.S. Patent Document Number	Kind Code[2] (if known)	Name of Patentee or Applicant of Cited Document	Date of Publication of Cited document MM-DD-YYYY
yc	A1	5,977,310		Namiki, et al.	November 2, 1999

FOREIGN PATENT DOCUMENTS

Examiner Initials'	Cite No.[1]	Foreign Patent Document Office[3]	Number[4]	Kind Code[5] (if known)	Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	T[6]
~~—~~	~~B1~~	~~EP 0 816 381~~ Duplicate for (892)				~~January 7, 1998~~	
yc	B2	WO 94/13322				June 23, 1994	
yc	B3	WO 93/23541				November 25, 1993	

NON PATENT LITERATURE DOCUMENTS

Examiner Initials'	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published	T[2]
yc	C1	Date, K., et al., FEBS Letters Vol. 420 (1997) pgs. 1-6	
~~—~~	~~C2~~	~~Date, K., et al., Oncogene (1998) Vol. 17 pgs. 3045-3054~~ Duplicate (892)	
yc	C3	Kuba, K., et al. Cancer Research (2000) Vol. 60, pgs. 6737-6743	
yc	C4	Uematsu Y., et al., Journal of Pharmaceutical Sciences (1/99), Vol. 88 No. 1, pgs. 131-135	
~~—~~	~~C5~~	~~Gaertner, H., et al., Bioconjugate Chem. (1996) Vol. 7 pgs. 38-44~~ Duplicate (892)	
yc	C6	Francis, G.E., et al., International Journal of Hematology, Vol. 68 (1998) pgs. 1-18	
	C7	Tsutsumi, Y. et al. Thrombosis & Haemostasis Vol. 77, No. 1 (1997) pgs. 168-173	
	C8	Tsutsumi, Y. et al., Proceedings of the National Academy of Sciences of the United States, Vol. 97, No. 15 (7/18/00) pgs. 8548-8553)	
	C9	Heinzerling, L., et al., Dermatology (2000) Vol. 201, pgs. 154-157	
	C10	Tsutsumi, Y. et al., Journal of Pharmacology & Experimental Therapeutics Vol. 278 No. 3 (1996) pgs. 1006-1011	
	C11	Reddy, R., et al. Annals of Pharmacotherapy Vol. 34, No. 7-8 (7/2000) pgs. 915-923	
	C12	Bailon, P., Bioconjugate Chem. Vol. 12 (02/16/2001) pgs. 195-202	
yc	C13	Parr, C., et al., Int. J. Cancer Vol. 85 (2000) pgs. 563-570	

Examiner Signature	Gyan Chandra	Date Considered	3/10/06

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Unique citation designation number. [2]See attached Kinds of U.S. Patent Documents. [3]Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4]For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6]Applicant is to place a check mark here if English language Translation or abstract is attached.

1: Int J Pept Protein Res. 1994 Feb;43(2):127-38.

Pegylated peptides. II. Solid-phase synthesis of amino-, carboxy- and side-chain pegylated peptides.

Lu YA, Felix AM.

Roche Research Center, Hoffmann-La Roche Inc., Nutley, New Jersey.

General procedures are presented for the site-specific pegylation of peptides at the NH2-terminus, side-chain positions (Lys or Asp/Glu) or COOH-terminus using solid-phase Fmoc/tBu methodologies. A model tridecapeptide fragment of interleukin-2, IL-2(44-56)-NH2, was chosen for this study since it possesses several trifunctional amino acids which serve as potential sites for pegylation. The pegylation reagents were designed to contain either Nle or Orn, which served as diagnostic amino acids for confirming the presence of 1 PEG unit per mole of peptide. NH2-Terminal pegylation was carried out by coupling PEG-CH2CO-Nle-OH to the free NH2-terminus of the peptide-resin. Side-chain pegylation of Lys or Asp was achieved by one of two pathways. Direct side-chain pegylation was accomplished by coupling with Fmoc-Lys(PEG-CH2CO-Nle)-OH or Fmoc-Asp(Nle-NH-CH2CH2-PEG)-OH, followed by solid-phase assemblage of the pegylated peptide-resin and TFA cleavage. Alternatively, allylic protective groups were introduced via Fmoc-Lys(Alloc)-OH or Fmoc-Asp (O-Allyl)-OH, and selectively removed by palladium-catalyzed deprotection after assemblage of the peptide-resin. Solid-phase pegylation of the side-chain of Lys or Asp was then carried out in the final stage with PEG-CH2CO-Nle-OH or H-Nle-NH-(CH2)2-PEG, respectively. COOH-Terminal pegylation was achieved through the initial attachment of Fmoc-Orn(PEG-CH2CO)-OH to the solid support, followed by solid-phase peptide synthesis using the Fmoc/tBu strategy. The pegylated peptides were purified by dialysis and preparative HPLC and were fully characterized by analytical HPLC, amino acid analysis, 1H-NMR spectroscopy and laser desorption mass spectrometry.

PMID: 8200730 [PubMed - indexed for MEDLINE]